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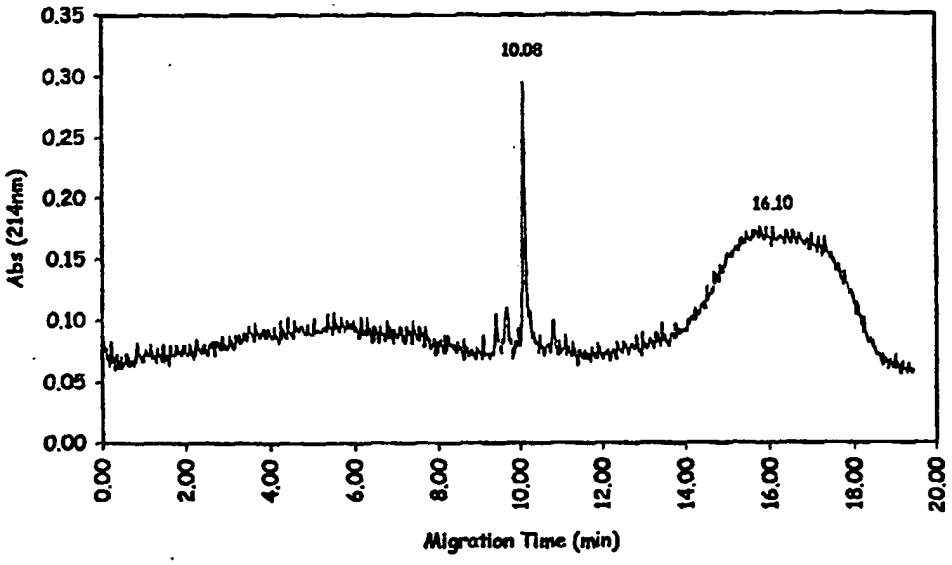

(57) Abstract

PEG-IFN-β conjugates, where a PEG moiety is covalently bound to Cys^{17} of human IFN-β, are produced by a process of site specific PEGylation with a thiol reactive PEGylating agent. A pharmaceutical composition and a method for treating infections, tumors and autoimmune and inflammatory diseases are also provided. The invention further relates to a method for the stepwise attachment of PEG moieties in series to a polypeptide, and more particularly to IFN-β.

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POLYOL-IFN-BETA CONJUGATES

CROSS-REFERENCE TO RELATED APPLICATION

The present application claims priority under 35 U.S.C. §119(e) from U.S. provisional application no. 60/083,339, the entire contents of which are hereby incorporated by reference.

FIELD OF THE INVENTION

The invention relates to polyol-IFN-β conjugates wherein a polyol unit is covalently bound to Cys^{17}. Further objects of the present invention are the process for their site-specific production as well as their use in the therapy, prognosis or diagnosis of bacterial infections, viral infections, autoimmune diseases and inflammatory diseases. The present invention further relates to a method for the stepwise attachment of two or more PEG moieties to a polypeptide.

BACKGROUND OF THE INVENTION

Human fibroblast interferon (IFN-β) has antiviral activity and can also stimulate natural killer cells against neoplastic cells. It is a polypeptide of about 20,000 Da induced by viruses and double-stranded RNAs. From the nucleotide sequence of the gene for fibroblast interferon, cloned by recombinant DNA technology, Derynk et al. (Nature, 285:542-547, 1980) deduced the complete amino acid sequence of the protein. It is 166 amino acid long.

Shepard et al. (Nature, 294:563-565, 1981) described a mutation at base 842 (Cys → Tyr at position 141) that abolished its anti-viral activity, and a variant clone with a deletion of nucleotides 1119-1121.

Mark et al. (Proc. Natl. Acad. Sci. U.S.A., 81(18):5662-5666, 1984) inserted an artificial mutation by replacing base 469 (T) with (A) causing an amino acid switch from Cys → Ser at position 17. The resulting IFN-β was reported to be as active as the 'native' IFN-β and stable during long-term storage (-70°C).

Covalent attachment of the hydrophilic polymer

polyethylene glycol, (PEG), also known as polyethylene oxide, (PEO), to molecules has important applications in biotechnology and medicine. In its most common form, PEG is a linear polymer having hydroxyl groups at each terminus:

$$HO{-}CH_2{-}CH_2O(CH_2CH_2O)_nCH_2CH_2{-}OH$$

This formula can be represented in brief as HO-PEG-OH, where it is meant that -PEG- represents the polymer backbone without the terminal groups:

"-PEG-" means "$-CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2-$

PEG is commonly used as methoxy-PEG-OH, (m-PEG), in which one terminus is the relatively inert methoxy group, while the other terminus is a hydroxyl group that is subject to chemical modification.

$$CH_3O{-}(CH_2CH_2O)_n{-}CH_2CH_2{-}OH$$

Branched PEGs are also in common use. The branched PEGs can be represented as $R(\text{-PEG-OH})_m$ in which R represents a central core moiety such as pentaerythritol or glycerol, and m represents the number of branching arms. The number of branching arms (m) can range from three to a hundred or more. The hydroxyl groups are subject to chemical modification.

Another branched form, such as that described in PCT patent application WO 96/21469, has a single terminus that is subject to chemical modification. This type of PEG can be represented as $(CH_3O{-}PEG{-})_pR{-}X$, whereby p equals 2 or 3, R represents a central core such as lysine or glycerol, and X represents a functional group such as carboxyl that is subject to chemical activation. Yet another branched form, the "pendant PEG", has reactive groups, such as carboxyl, along the PEG backbone rather than at the end of PEG chains.

In addition to these forms of PEG, the polymer can also be prepared with weak or degradable linkages in the backbone. For example, Harris has shown in U.S. Patent Application 06/026,716 that PEG can be prepared with ester linkages in the polymer backbone that are subject to hydrolysis. This hydrolysis results in cleavage of the polymer into fragments of lower molecular weight, according to the reaction scheme:

$$-PEG-CO_2-PEG- + H_2O \rightarrow -PEG-CO_2H + HO-PEG-$$

According to the present invention, the term polyethylene glycol or PEG is meant to comprise all the above described derivatives.

The copolymers of ethylene oxide and propylene oxide are closely related to PEG in their chemistry, and they can be used instead of PEG in many of its applications. They have the following general formula:

$$HO-CH_2CHRO(CH_2CHRO)_nCH_2CHR-OH$$

wherein R is H or CH_3.

PEG is a useful polymer having the property of high water solubility as well as high solubility in many organic solvents. PEG is also non-toxic and non-immunogenic. When PEG is chemically attached (PEGylation) to a water insoluble compound, the resulting conjugate generally is water soluble, as well as soluble in many organic solvents.

PEG-protein conjugates are currently being used in protein replacement therapies and for other therapeutic uses. For example, PEGylated adenosine deaminase (ADAGEN®) is being used to treat severe combined immunodeficiency disease (SCIDS), PEGylated L-asparaginase (ONCAPSPAR®) is being used to treat acute lymphoblastic leukemia (ALL), and PEGylated interferon-α (INTRON(R) A) is in Phase III trials for treating hepatitis C.

For a general review of PEG-protein conjugates with clinical efficacy see N.L. Burnham, Am. J. Hosp. Pharm., 15:210-218, 1994.

A variety of methods have been developed to PEGylate proteins. Attaching PEG to reactive groups found on the protein is typically done utilizing electrophilically activated PEG derivatives. Attaching PEG to the α- and ε-amino groups found on lysine residues and the N-terminus results in a conjugate consisting of a mixture of products.

Generally, such conjugates consist of a population of the several PEG molecules attached per protein molecule ("PEGmers") ranging from zero to the number of amino groups in the protein. For a protein molecule that has been singly modified, the PEG unit may be attached at a number of different amine sites.

This type of non-specific PEGylation has resulted in a number of conjugates that become almost inactive. Reduction of activity is typically caused by shielding the protein's active binding domain as is the case with many cytokines and antibodies. For example, Katre et al. in U.S. Patent 4,766,106 and U.S. Patent 4,917,888 describe the PEGylation of IFN-β and IL-2 with a large excess of methoxy-polyethylene glycolyl N-succinimidyl glutarate and methoxy-polyethylene glycolyl N-succinimidyl succinate. Both proteins were produced in microbial host cells, which allowed the site-specific mutation of the free cysteine to a serine. The mutation was necessary in microbial expression of IFN-β to facilitate protein folding. In particular, the IFN-β used in these experiments is the commercial product Betaseron®, in which Cys^{17} residue is replaced with a serine. Additionally, the absence of glycosylation reduced its solubility in aqueous solution. Non-specific PEGylation resulted in increased solubility, but a major problem was the reduced level of activity and yield.

European Patent Application EP 593 868, entitled PEG-Interferon Conjugates, describes the preparation of PEG-IFN-α conjugates. However, the PEGylation reaction is not site-specific, and therefore a mixture of positional isomers of PEG-IFN-α conjugates are obtained (see also Monkarsh et al., ACS Symp. Ser., 680:207-216, 1997).

Kinstler et al. in European Patent Application EP 675 201 demonstrated the selective modification of the N-terminal residue of megakaryocyte growth and development factor (MGDF) with mPEG-propionaldehyde. This allowed for reproducible PEGylation and pharmacokinetics from lot to lot. Gilbert et al. in U.S. Patent 5,711,944 demonstrated that PEGylation of IFN-α with an optimal level of activity could be produced. In this instance a laborious purification step was needed to obtain the optimal conjugate.

The majority of cytokines, as well as other proteins, do not possess a specific PEG attachment site and, apart from the examples mentioned above, it is very likely that some of the isomers produced through the PEGylation reaction be partially or

totally inactive, thus causing a loss of activity of the final mixture.

Site-specific mono-PEGylation is thus a desirable goal in the preparation of such protein conjugates.

Woghiren et al. in Bioconjugate Chem., 4(5):314-318, 1993, synthesized a thiol-selective PEG derivative for such a site-specific PEGylation. A stable thiol-protected PEG derivative in the form of an orthopyridyl disulfide reactive group was shown to specifically conjugate to the free cysteine in the protein, papain. The newly formed disulfide bond between papain and PEG could be cleaved under mild reducing conditions to regenerate the native protein.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicants at the time of filing and does not constitute an admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

In the present invention, polyol-IFN-β conjugates, and particularly PEG-IFN-β conjugates, are provided wherein a polyol unit is covalently bound to Cys^{17}. The specific conjugation is obtained by allowing a thiol-reactive polyol agent to react with the Cys^{17} residue in IFN-β. Such conjugates are expected to show increased effectiveness *in vivo*. The aim is to obtain increased solubility at neutral pH, increased stability (decreased aggregation), decreased immunogenicity, and no loss of activity with respect to 'native' IFN-β. The results of such conjugation would decrease the number of doses for an intended effect, simplify and stabilize the formulation of a pharmaceutical composition, and possibly increase the long-term efficacy.

The present invention further provides a method for the stepwise attachment of PEG moieties in series to a polypeptide.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the Capillary Electrophoresis (CE) graph of the PEG-IFN-β conjugate prior to purification.

Figures 2A-2C show the purification of the PEG-IFN-β conjugate carried out by size exclusion chromatography (Superose 12): Fig. 2A - first pass; Fig. 2B - second pass; Fig. 2C - third pass.

Figure 3 shows the SDS-PAGE chromatography of purified PEG-IFN-β conjugate from the third pass of chromatography. Lanes 1 and 4 are protein molecular weight standards, lane 2 is "native" IFN-β, and lane 3 is PEG-IFN-β conjugate.

Figure 4 reports the Capillary Electrophoresis (CE) graph of purified PEG-IFN-β conjugate in which IFN-β is PEGylated with mPEG-$OPSS_{5k}$.

Figure 5 reports the MALDI MS spectrum of purified PEG-IFN-β conjugate.

Figure 6 shows a comparison between the anti-viral activity of "native" IFN-β and of PEG-IFN-β conjugate. WISH cells were incubated with indicated concentrations of IFN-β samples for 24 hours prior to challenge with cytopathic dose of vesicular stomatitis virus. The cytopathic effect was determined after an additional 48 hours by MTT conversion.

Figure 7 shows the binding profile of IFN-β and PEG-IFN in Daudi cells.

Figure 8 shows the pharmokinetic profile of IFN-β and PEG-IFN in mice following intravenous administration. The dotted lines indicate assay LOQ for each standard curve.

Figure 9 shows the pharmokinetic profile of IFN-β and PEG-IFN in mice following subcutaneous administration. The dotted lines indicate assay LOQ for each standard curve.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the discovery that the attachment of a polyol moiety, more specifically a PEG moiety, to the Cys^{17} residue of human IFN-β unexpectedly increased (or at least retained and did not result in a decrease) the IFN-β biological activity from that of native

human interferon-β. Thus, not only does IFN-β with a polyol moiety attached to the Cys^{17} residue exhibit the same or increased IFN-β biological activity but this polyol-IFN-β conjugate also provides the desirable properties conferred by the polyol moiety, such as increased solubility.

"IFN-β", as used herein, means human fibroblast interferon, as obtained by isolation from biological fluids or as obtained by DNA recombinant techniques from prokaryotic or eukaryotic host cells as well as its salts, functional derivatives, precursors and active fractions, provided that they contain the cysteine residue appearing at position 17 in the naturally occurring form.

The polyol moiety in the polyol-IFN-β conjugate according to the present invention can be any water-soluble mono- or bifunctional poly(alkylene oxide) having a linear or branched chain. Typically, the polyol is a poly(alkylene glycol) such as poly(ethylene glycol) (PEG). However, those of skill in the art will recognize that other polyols, such as, for example poly (propylene glycol) and copolymers of polyethylene glycol and polypropylene glycol, can be suitably used.

As used herein, the term "PEG moiety" is intended to include, but is not limited to, linear and branched PEG, methoxy PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, dendrimer PEG, copolymers of PEG and one or more polyols, and copolymers of PEG and PLGA (poly(lactic/glycolic acid)).

The definition "salts" as used herein refers both to salts of the carboxyl-groups and to the salts of the amino functions of the compound obtainable through known methods. The salts of the carboxyl-groups include inorganic salts as, for example, sodium, potassium, calcium salts and salts with organic bases as those formed with an amine as triethanolamine, arginine or lysine. The salts of the amino groups included for example, salts with inorganic acids as hydrochloric acid and with organic acids as acetic acid.

The definition "functional derivatives" as herein used refers to derivatives which can be prepared from the functional groups present on the lateral chains of the amino acid moieties or on the terminal N- or C- groups according to known methods

and are included in the present invention when they are pharmaceutically acceptable, i.e., when they do not destroy the protein activity or do not impart toxicity to the pharmaceutical compositions containing them. Such derivatives include for example esters or aliphatic amides of the carboxyl-groups and N-acyl derivatives of free amino groups or O-acyl derivatives of free hydroxyl-groups and are formed with acyl-groups as for example alcanoyl- or aroyl-groups.

The "precursors" are compounds which are converted into IFN-β in the human or animal body.

As "active fractions" of the protein, the present invention refers to any fragment or precursor of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example, residues of sugars or phosphates, or aggregates of the polypeptide molecule when such fragments or precursors show the same activity of IFN-β as medicament.

The conjugates of the present invention can be prepared by any of the methods known in the art. According to an embodiment of the invention, IFN-β is reacted with the PEGylating agent in a suitable solvent and the desired conjugate is isolated and purified, for example, by applying one or more chromatographic methods.

"Chromatographic method" means any technique that is used to separate the components of a mixture by their application on a support (stationary phase) through which a solvent (mobile phase) flows. The separation principles of the chromatography are based on the different physical nature of stationary and mobile phase.

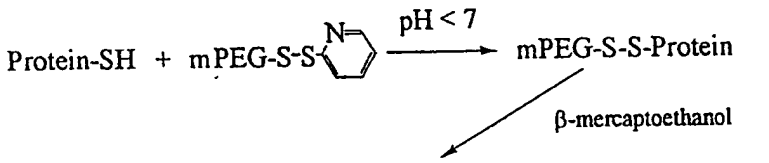
Some particular types of chromatographic methods, which are well-known in the literature, include: liquid, high pressure liquid, ion exchange, absorption, affinity, partition, hydrophobic, reversed phase, gel filtration, ultrafiltration or thin-layer chromatography.

The "thiol-reactive PEGylating agent", as used in the present application, means any PEG derivative which is capable of reacting with the thiol group of the cysteine residue. It can be, for example, PEG containing a functional group such as

orthopyridyl disulfide, vinylsulfone, maleimide, iodoacetimide, and others. According to a preferred embodiment of the present invention, the thiol-reactive PEGylating agent is the orthopyridyl disulfide (OPSS) derivative of PEG.

The PEGylating agent is used in its mono-methoxylated form where only one terminus is available for conjugation, or in a bifunctional form where both termini are available for conjugation, such as for example in forming a conjugate with two IFN-β covalently attached to a single PEG moiety. It has preferably a molecular weight between 500 and 100,000.

A typical reaction scheme for the preparation of the conjugates of the invention is presented below:



$$\text{Protein-S-H} + \text{mPEG-S-H} + \text{HOCH}_2\text{CH}_2\text{-S-S-CH}_2\text{CH}_2\text{OH}$$

The second line of the above scheme reports a method for cleaving the PEG-protein linkage. The mPEG-OPSS derivative is highly selective for free sulphydryl groups and reacts rapidly under acidic pH conditions where the IFN-β is stable. The high selectivity can be demonstrated from the reduction of the conjugate to the native form of IFN-β and PEG.

The disulfide bond that is produced between the protein and PEG moieties has been shown to be stable in the circulation, but it can be reduced upon entering the cell environment. Therefore it is expected that this conjugate, which does not enter the cell, will be stable in the circulation until it is cleared.

It should be noted that the above reaction is site-specific because the other two Cys residues appearing at positions 31 and 141 in the naturally occurring form of human IFN-β do not react with the thiol-reactive PEGylating agent since they form a disulfide bridge.

The present invention is also directed to a method for the stepwise attachment of two or more PEG moieties to a polypeptide. This method is based upon the recognition that a low molecular weight activated PEG reacts more completely with a sterically hindered reaction site on a protein than does a high molecular weight activated PEG. PEG-modification of expensive therapeutic proteins must be cost effective in order for the production of the PEG conjugate to be practical. In addition, in order to reduce glomerular filtration and optimize the pharmacological properties of the PEG-protein conjugate, the conjugate should have an effective size equivalent to that of a protein with a molecular weight of 70 kDa. This means that for a site specific modification where one PEG will be attached, a PEG derivative having a molecular weight of greater than 20 kDa is preferably attached. If the site of modification is sterically crowded, the reactive group on the large PEG moiety may have difficulty reaching the modification site and thus will lead to low yields. A preferred method of PEGylating a polypeptide according to the present invention increases the yield of site-specific PEGylation by first attaching a small hetero or homobifunctional PEG moiety that, due to its relatively smaller size, can react with sterically crowded sites. Subsequent attachment of a large molecular weight PEG derivative to the small PEG results in high yield of the desired PEGylated protein.

The method for stepwise attachment of two or more PEG moieties in series to a polypeptide according to the present invention includes attaching a low molecular weight heterbifunctional or homobifunctional PEG moiety first to the polypeptide and then attaching a monofunctional or bifunctional PEG moiety to the free terminus of the low molecular weight PEG moiety that is attached to the polypeptide. Following the stepwise attachment of two or more PEG moieties in series to a polypeptide, which polypeptide is preferably IFN-β and where Cys^{17}, located in a sterically crowded site, is the preferred site of PEG attachment, the PEG-polypeptide conjugate can be purified using one or more of the purification techniques such as ion exchange chromatography, size exclusion chromatography,

hydrophobic interaction chromatography, affinity chromatography, and reverse phase chromatography.

The low molecular weight PEG moiety has the formula:

$$W\text{-}CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2\text{-}X,$$

where W and X are groups that independently react with an amine, sulfhydryl, carboxyl or hydroxyl functional group to attach the low molecular weight PEG moiety to the polypeptide. W and X are preferably independently selected from orthopyridyl disulfide, maleimides, vinyl sulfones, iodoacetamides, amines, thiols, carboxyls, active esters, benzotriazole carbonates, p-nitrophenol carbonates, isocyanates, and biotin. The low molecular weight PEG moiety preferably has a molecular weight in the range of about 100 to 5,000 daltons.

The monofunctional or bifunctional PEG moiety for attachment to the free terminus of a low molecular weight PEG that is attached to the polypeptide has preferably a molecular weight in the range of about 100 daltons to 200 kDa and is preferably a methoxy PEG, branched PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, or dendrimer PEG. The monofunctional or bifunctional PEG furthermore has the formula:

$$Y\text{-}CH_2CH_2O(CH_2CH_2O)_mCH_2CH_2\text{-}Z,$$

where Y is reactive to a terminal group on the free terminus of the low molecular weight PEG moiety that is attached to the polypeptide and Z is $-OCH_3$ or a group reactive with to form a bifunctional conjugate.

The PEG-polypeptide conjugate produced by the above method for stepwise attachment of two or more PEG moieties can be used to produce a medicament or pharmaceutical composition for treating diseases or disorders for which the polypeptides is effective as an active ingredient.

Another object of the present invention is to provide the conjugates in substantially purified form in order for them to be suitable for use in pharmaceutical compositions, as active

ingredient for the treatment, diagnosis or prognosis of bacterial and viral infections as well as autoimmune, inflammatory diseases and tumors. Such pharmaceutical compositions represent a further object of the present invention.

Non-limiting examples of the above-mentioned diseases include: septic shock, AIDS, rheumatoid arthritis, lupus erythematosus and multiple sclerosis.

Further embodiments and advantages of the invention will be evident in the following description.

An embodiment of the invention is the administration of a pharmacologically active amount of the conjugates of the invention to subjects at risk of developing one of the diseases reported above or to subjects already showing such pathologies.

Any route of administration compatible with the active principle can be used. Parenteral administration, such as subcutaneous, intramuscular or intravenous injection is preferred. The dose of the active ingredient to be administered depends on the basis of the medical prescriptions according to age, weight and the individual response of the patient.

The dosage can be between 10 μg and 1 mg daily for an average body weight of 75 kg, and the preferable daily dose is between 20 μg and 200 μg.

The pharmaceutical composition for parenteral administration can be prepared in an injectable form comprising the active principle and a suitable vehicle. Vehicles for the parenteral administration are well known in the art and include, for example, water, saline solution, Ringer solution and/or dextrose. The vehicle can contain small amounts of excipients in order to maintain the stability and isotonicity of the pharmaceutical preparation. The preparation of the solutions can be carried out according to the ordinary modalities.

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention.

EXAMPLE 1: Preparation of PEG-IFN-β Conjugate

Modification of IFN-β with $mPEG_{5k}$-OPSS

Recombinant human IFN-β, stable at a concentration of 0.37 mg/ml in 50 mM sodium acetate buffer, pH 3.6, was used for the preparation of a PEG-IFN-β conjugate. Approximately 1.0 ml of 6 M urea was added to 2 ml of IFN-β at a concentration of 0.37 mg/ml (0.74 mg, 3.7×1^{-8} moles). $mPEG_{5K}$-OPSS was added in a molar excess of 50 moles to one mole of IFN-β and the two were allowed to react in a polypropylene vial for either 2 hours at 37°C or 1 hour at 50°C. The reaction mixture was analyzed with Capillary Electrophoresis (CE) graph to determine the extent of PEG-IFN-β conjugate formation by the PEGylation reaction prior to any purification (Fig. 1). A typical yield for this reaction is 50% PEG-IFN-β. The reaction products were filtered from the reaction mixture with a 0.22 mm syringe filter and the filtered solution was then loaded onto a size exclusion column (either Superose 12 or Superdex 75, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. Fig. 2A shows the elution profile from the purification of the PEG-IFN-β conjugate on a Superose 12 size exclusion chromatography column. The peaks were collected and analyzed with SDS-PAGE (Fig. 3). The fractions containing the PEG-IFN-β conjugate were pooled together and concentrate was then reloaded to the same size exclusion column to further purify the PEG-IFN-β conjugate due to the close proximity of the "native" IFN-β peak (Fig. 2B). This procedure was repeated (third pass) to ensure purity (Fig. 2C). Fig. 4 and Fig. 5 show the Capillary Electrophoresis graph and the MALDI MS spectrum, respectively, of the purified PEG-IFN-β conjugate.

Modification of IFN-β with $mPEG_{30K}$-OPSS

Recombinant human IFN-β was provided is stable in solution at 0.36 mg/ml in 50 mM sodium acetate buffer, pH 3.6.

Approximately 36 mg of $mPEG_{30K}$-OPSS in 3 ml deionized H_2O was added to 3 ml of IFN-β at 0.36 mg/ml (1.08 mg, $4.9\text{-}x10^{-8}$ moles) and the two were allowed to react in a polypropylene vial for 2 hours at 50°C. The reaction mixture was analyzed with capillary electrophoresis for extent of modification. Typical yields for this reaction are <30%. The solution was then loaded onto a size exclusion column (Superose 12, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE for their contents.

EXAMPLE 2: Biological Activity of the PEG-IFN-β Conjugate

To assess the effects of PEGylation on the anti-viral activity of human recombinant IFN-β, human WISH amniotic cells were preincubated with either freshly prepared IFN-β (same lot as used for PEGylation) or PEG-IFN-β conjugate. The IFN-β-mediated anti-viral activity, as measured by the WISH-VSV cytopathicity assay, was determined according to an anti-viral WISH bioassay developed based on the protocol of Novick et al., J. Immunol., 129:2244-2247 (1982). The materials used in this WISH assay is as follows:

WISH cells (ATCC CCL 25)

Vesicular Stomatitis Virus stocks (ATCC V-520-001-522), stored at -70°C

IFN-β, human recombinant, InterPharm Laboratories LTD (32,075-type, Batch # 205035), 82 x 10^6 IU/ml, specific activity: 222 x 10^6 IU/mg

PEG-IFN-β conjugate as prepared in Example 1 and maintained in PBS, pH 7.4

WISH Growth medium (MEM high glucose with Earls salts + 10% FBS + 1.0% L-glutamine + Penicillin/Streptomycin (100 U/ml, 100 μg/ml)

WISH Assay medium (MEM high glucose with Earls salts + 5% FBS + 1.0% L-glutamine + Penicillin/Streptomycin (100 U/ml, 100 μg/ml)

MTT at 5 mg/ml in PBS, stored at minus 70°C.

The protocol for the WISH assay is as follows:

Dilute the IFN-β samples to 2X the starting concentration in WISH assay medium.

Make three-fold dilutions of IFN-β samples in WISH assay medium in flat-bottomed 96-well plate so that each well contains 50 μl of diluted IFN-β sample (some control wells receive 50 μl of WISH assay medium only).

Harvest log growth phase WISH cells with trypsin/EDTA solution, wash in WISH assay medium, and bring to a final concentration of 0.8×10^6 cells/ml.

Add 50 μl of WISH cell suspension (4×10^4 cells per well) to each well. Final concentration of IFN-β exposed to the cells is now 1X.

After incubation for 24 hours in a 5% CO_2 humidified incubator, 50 μl of a 1:10 dilution (in WISH assay medium) of VSV stock (a dose predetermined to lyse 100 percent of WISH cells within 48 hours) is added to all wells except for the no virus control wells (these receive an equal volume of assay medium only).

After an additional 48 hours, 25 μl of MTT solution is added to all wells, after which plates are incubated for an additional 2 hours in an incubator.

The contents of the wells are removed by plate inversion, and 200 μl of 100% ethanol is added to the wells.

After 1 hour, the plates are read at 595 nm using the Soft max Pro software package and Spectramax spectrophotometer system (Molecular Devices).

Table 1. Antiviral Activity of PEGylated and Mock-PEGylated IFN-beta Samples

IFN-beta Sample*	EC_{50}**
PEG-IFN-β conjugate	3.9 +/- 0.7 pg/ml
IFN-β	16.4 +/- 1.0 pg/ml

* Stock concentrations of IFN-β in samples determined by amino acid analysis.

** EC_{50} (+/- S.D.) was determined by software program Microcal Origin 4.1

As demonstrated in Fig. 6 and Table 1 above, the PEG-IFN-β conjugate maintained a level of anti-viral activity superior to that of the freshly prepared parental lot of IFN-β.

The observation that the PEG-IFN-β conjugate has approximately 4-fold higher bioactivity than that of freshly prepared IFN-β may be also a consequence of the increased stability of the PEG-IFN-β conjugate with respect to the "native" IFN-β after addition of WISH cell assay medium.

EXAMPLE 3: In Vitro Assays of the Relative Activity of PEG-IFN samples

Relative bioactivity of PEG[30 kD]-IFN-β and PEG[2 X 20 kD]-IFN-β was determined by WISH assay using the standard protocol described in Example 2 (Table 2). Three independent assays were performed by three different individuals at separate times.

Table 2. Relative antiviral activity of PEG-IFN-β

	Relative Interferon Activity* (from three studies)			
Sample	Assay 1	Assay 2	Assay 3	Average (S.D.)
PEG[30kD]-IFN-β	3.2 X higher	3.1 X higher	1.8 X higher	3.0 X (0.78) higher
PEG[2 x 20kD]-IFN-β	4.2 X higher	1.3 X higher	0.85 X higher	2.1 X (1.8) higher

*EC50 doses compared with standard IFN-β included in each assay.

**Comparison based on IFN-β concentration of 330 ug/ml. Stock concentrations of PEG[30 kD]-IFN-β (5.41 ug/ml) and PEG[2 x 20 kD]-IFN-β (6.86 ug/ml) were determined by AAA.

The binding of PEG-IFN-β to its receptor on cells was evaluated in the presence of a fixed amount of ^{125}I-IFN-α2a. IFN-α2a was radiolabeled with ^{125}I using the chloramine T method. The ^{125}I bound IFNα2a was removed from free iodine by running the reactants through a Sephadex G25 column and pooling the protein containing fractions (Pharmacia). ^{125}I-IFN-α2a was quantified by an IFN-α2a ELISA assay (Biosource, USA) and the specific activity was determined. Daudi cells grown in the exponential phase of growth were harvested and 2 x 10^6 cells were incubated with 0.5 nM ^{125}I-IFN-α2a for 3 hours at room temperature in the presence of different concentrations of PEG-IFN-β or IFN-α2a diluted in an assay buffer which is RPMI 1640 containing 2%

fetal bovine serum and 0.1% sodium azide. At the end of the incubation, the cells were spun through a layer of phthalate oil and the cell bound radioactivity was counted on the gamma counter. Furthermore, the binding of PEG[30kD]-IFN-β and PEG[2 x 20kD]-IFN-β to the receptor were very similar or close to the binding activity of IFN-β as shown in Fig. 7.

In addition, relative activity was determined in a Daudi cell (human B cell lymphoma) anti-proliferation assay (Table 3). All IFNs were made at a 2x concentration of 200 ng/ml. Samples were diluted three-fold down the length of the plate at a final volume of 100 ul. 1 x 10^5 cells/well (100 µls) were added to each well and incubated for a total of 72 hours at 37°C in CO_2 humidified incubator. After 48 hours, tritiated (^3H) thymidine were added at 1 µCi/well in 20 ul. At the end of the 72 hour incubation, the plate was harvested with the Tomtek Plate Harvester. The results shown in Table 3 indicate that no detectable loss of IFN activity was observed from PEGylation. In fact, the activity was found to be somewhat higher than free IFN-β. This may be due to the formation of inactive aggregates in the free IFN or to the differences in quantitation methods (amino acid analysis for PEG-IFN samples and RP-HPLC for IFN-β).

Table 3. Daudi Anti-Proliferation Assay

	IC_{50}dose*	Fold increase vs IFN
IFN-β (Plate 1)	1153.1	–
PEG[30kD]-IFN (71 A)	695.6	1.6X
IFN-β (Plate 2)	1005.8	–
PEG[40kD]-IFN (71 B)	629.4	1.7X

*pg/ml

EXAMPLE 4: Pharmacokinetic Studies in Mice

Intravenous Administration

Mice were injected with 100 ng of IFN-β, PEG[30kD]-IFN-β or PEG[2 X 20 kD]-IFN-β and blood was removed at indicated times thereafter. Serum concentrations of IFN-β were determined

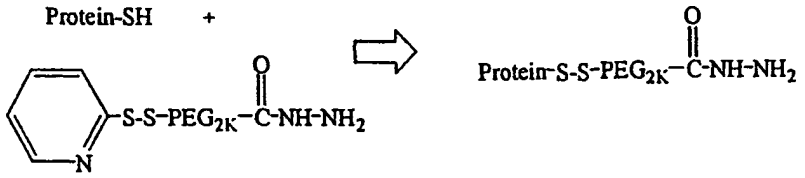
by IFN-β-specific ELIAS (Toray Industries) and the results are shown in Fig. 8. Twenty-eight female B6D2F1 strain mice (6-8 wks) (approximately 20g each) were separated into four groups as follows: Group 1 contained nine mice injected with a 200 ul single bolus of 500 ng/ml human IFN-β (final dose of 100 ng/ mouse); Group 2 (nine mice) received 200 ul of an equivalent mass of PEG30kD-IFN-β; Group 3 received 200 ul of an equivalent mass of PEG(2 x 20 kD)-IFN-β; and Group 4 is a group of three uninjected mice serving as a negative control. Blood samples (approximately 200 ul/sample) were collected at nine indicated times by disruption of the retro-orbital venous plexus with a capillary tube. Blood samples were allowed to clot for 1 hr at room temperature, rimmed and microcentrifuged. Sera removed therefrom were stored at -70°C until all samples were collected. Sera were assayed for the presence of bioactive human IFN-β using the Toray assay. The results indicate that the area under the curve (AUC) is markedly enhanced in the PEG-IFN samples versus free IFN-beta and that PEG-IFN samples versus free IFN-β and that PEG[2 X 20 kD]-IFN-β is superior to the PEG[30 kD]-IFN-β.

Subcutaneous Administration

Mice were injected subcutaneously with IFN-β and PEG-IFN (100 ng/mouse). Figure 9 demonstrates that the total area under the curve (AUC) is dramatically enhanced for the PEG-IFN samples as compared with free IFN-β. The pharmacokinetic studies are consistent with the PEG-IFN samples having a longer half-life and increased AUC.

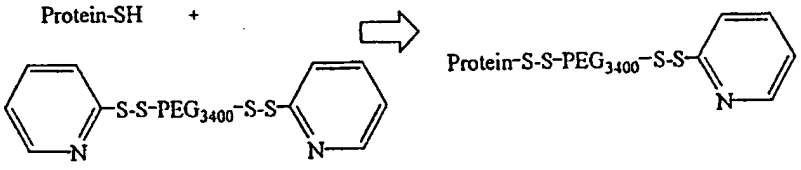
Example 5: Attachment of Low Molecular Weight PEG Moiety to Polypeptide

Tagging Interferon-beta with OPSS-PEG_{2k}-Hydrazide



Recombinant human interferon-β was provided in solution at 0.33 mg/ml in 50mM sodium acetate buffer, pH 3.8. Approximately 3.6 mg (40 mole excess to moles of protein) of the heterobifunctional PEG reagent, OPSS-PEG_{2k}-hydrazide, in 2 ml deionized water was added to 3 ml of IFN-β at 0.33 mg/ml (0.99 mg) and the two were allowed to react in a polypropylene vial for 1 hour at 45°C. The reaction mixture was then analyzed with capillary electrophoresis to determine the extent of modification. Typical yields ranged from 90-97% that depended on the purity of the interferon β and PEG reagent. The solution was next loaded onto a size exclusion column (Superdex 75, Pharmacia) and eluted with 5 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE. The monoPEGylated interferon-β fractions were pooled together than used in a further modification step with high molecular weight PEG.

Tagging Interferon-β with $(OPSS)_2$-PEG_{3400}

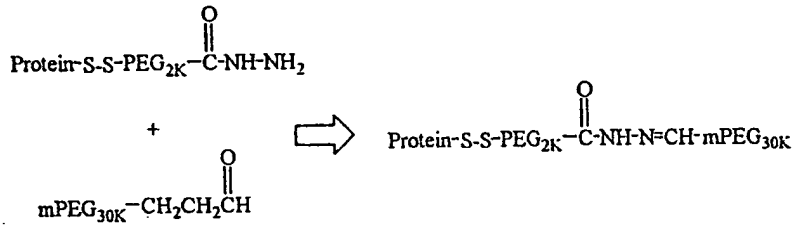


Recombinant human interferon-β was provided in solution at 0.33 mg/ml in 50 mM sodium acetate buffer, pH 3.8. Approximately 6.1 mg (40 mole excess to moles of protein) of the homobifunctional PEG reagent, $(OPSS)_2$-PEG_{3400}, in 2 ml deionized water was added to 3 ml of interferon-β at 0.33 mg/ml (0.99 mg) and the two were allowed to react in a polypropylene vial for 2 hours at 50°C. The reaction was monitored with non-reducing SDS-PAGE and the final reaction mixture was analyzed with capillary electrophoresis to determine the extent of modification. Typical modifications for this reaction with interferon-β were >95%. The solution was then loaded onto a

size exclusion column (Superdex 75, Pharmacia) and eluted with 50 mM sodium phosphate, 150 mM NaCl, pH 7.0 buffer. The peaks were collected and analyzed with SDS-PAGE for their contents. The monoPEGylated interferon-β fractions were combined.

EXAMPLE 6: Attachment of Second PEG Moiety to Low Molecular Weight PEGylating Polypeptide

Modification of IFN-S-S-PEG_{2k}-Hydrazide with $mPEG_{30k}$-Aldehyde (ALD)



To the combined fractions of IFN-S-S-PEG_{2k}-Hydrazide in Example 5 was added $mPEG_{30k}$-ALD in a 20 mole excess to protein. The reaction was conducted at room temperature (25°C) for 4 hours and a sample was added to a size exclusion column (Superose 6, Pharmacia) to determine modification yield. The modification yield of this reaction was typically >80% depending upon the purity of the PEG reagent and reaction conditions.

Having now fully described this invention, it will be appreciated that by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the

essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

WHAT IS CLAIMED IS:

1. A polyol-interferon-β conjugate having a polyol moiety covalently bound to Cys^{17} of human interferon-β.

2. The polyol-interferon-β conjugate according to claim 1, wherein said polyol moiety is a polyalkylene glycol moiety.

3. The polyol-interferon-β conjugate according to claim 2, wherein said polyalkylene glycol moiety is a polyethylene glycol (PEG) moiety.

4. The polyol-interferon-β conjugate according to any of claims 1-3, wherein the polyol-interferon-β conjugate has the same or higher interferon-β activity as native human interferon-β.

5. A process for producing the polyol-interferon-β conjugate of claim 1, comprising the steps of:

reacting interferon-β with a thiol-reactive polyol agent to site specifically and covalently attach a polyol moiety to Cys^{17} of human interferon-β to produce a polyol-interferon-β conjugate; and

recovering the produced polyol-interferon-β conjugate.

6. The process according to claim 5, wherein the thiol-reactive polyol agent is a thiol-reactive PEGylating agent.

7. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is mono-methoxylated.

8. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is bifunctional.

9. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is a polyol derivative having a functional group selected from the group consisting of orthopyridyl disulfide, vinyl sulfone, maleimide, and iodoacetimide.

10. The process according to either claim 5 or claim 6, wherein the thiol-reactive polyol agent is an orthopyridyl disulfide derivative of a mono-methoxylated polyol.

11. The process according to claim 5, wherein the reacting step is carried out at an acidic pH where interferon-β is stable.

12. A pharmaceutical composition, comprising a polyol-interferon-β conjugate according to any one of claims 1-3, as an active ingredient, and a pharmaceutically acceptable carrier, excipient or auxiliary agent.

13. A method for treating infections, tumors and autoimmune and inflammatory diseases, comprising administering an effective amount of the pharmaceutical composition according to claim 12 to a subject in need thereof.

14. A method for stepwise attachment of polyethylene glycol (PEG) moieties in series to a polypeptide, comprising the steps of:

reacting a polypeptide with a low molecular weight heterobifunctional or homobifunctional PEG moiety having the following formula:

$$W\text{-}CH_2CH_2O(CH_2CH_2O)_nCH_2CH_2\text{-}X,$$

where W and X are groups that independently react with an amine, sulfhydryl, carboxyl or hydroxyl functional group to attach the low molecular weight PEG moiety to the polypeptide; and

reacting the low molecular weight PEG moiety attached to the polypeptide with a monofunctional or bifunctional PEG moiety to attach the monofunctional or bifunctional PEG moiety to a free terminus of the low molecular weight PEG moiety and form a PEG-polypeptide conjugate.

15. The method according to claim 14, wherein the monofunctional or bifunctional PEG moiety has the following formula:

$$Y\text{-}CH_2CH_2O(CH_2CH_2O)_mCH_2CH_2\text{-}Z,$$

wherein Y is reactive to a terminal group on the free terminus of the low molecular weight PEG moiety attached to the polypeptide and Z is $-OCH_3$ or a group reactive with X to form a bifunctional conjugate.

16. The method according to claim 15, wherein the monofunctional or bifunctional PEG moiety is methoxy PEG, branched PEG, hydrolytically or enzymatically degradable PEG, pendant PEG, or dendrimer PEG.

17. The method according to claim 14, wherein W and X are independently selected from the group consisting of orthopyridyl disulfide, maleimides, vinylsulfones, iodoacetamides, hydrazides, aldehydes, succinimidyl esters, epoxides, amines, thiols, carboxyls, active esters, benzotriazole carbonates, p-nitrophenol carbonates, isocyanates, and biotin.

18. The method according to claim 14, wherein the low molecular weight PEG moiety has a molecular weight in a range of about 100 to 5,000 daltons.

19. The method according to claim 14, wherein the monofunctional or bifunctional PEG moiety has a molecular weight in a range of about 100 daltons to 200 kilodaltons.

20. The method according to claim 14, wherein the low molecular weight PEG moiety and/or the monofunctional or bifunctional PEG moiety is a copolymer of polyethylene glycol.

21. The method according to claim 20, wherein the copolymer of polyethylene glycol is selected from the group consisting of polyethylene glycol/polypropylene glycol copolymers and polyethylene glycol/poly(lactic/glycolic acid) copolymers.

22. The method according to claim 14, further comprising a step of purifying the PEG-polypeptide conjugate following the stepwise attachment of two PEG moieties in series to a polypeptide.

23. The method according to claim 22, wherein said step of purifying comprises one or more purification techniques selected from the group consisting of ion exchange chromatography, size exclusion chromatography, hydrophobic interaction chromatography, affinity chromatography, and reverse phase chromatography.

24. The method according to any one of claim 14-23, wherein the polypeptide is interferon-β.

25. Use of the PEG-polypeptide conjugates produced by the method according to any one of claims 14-23 as a medicament.

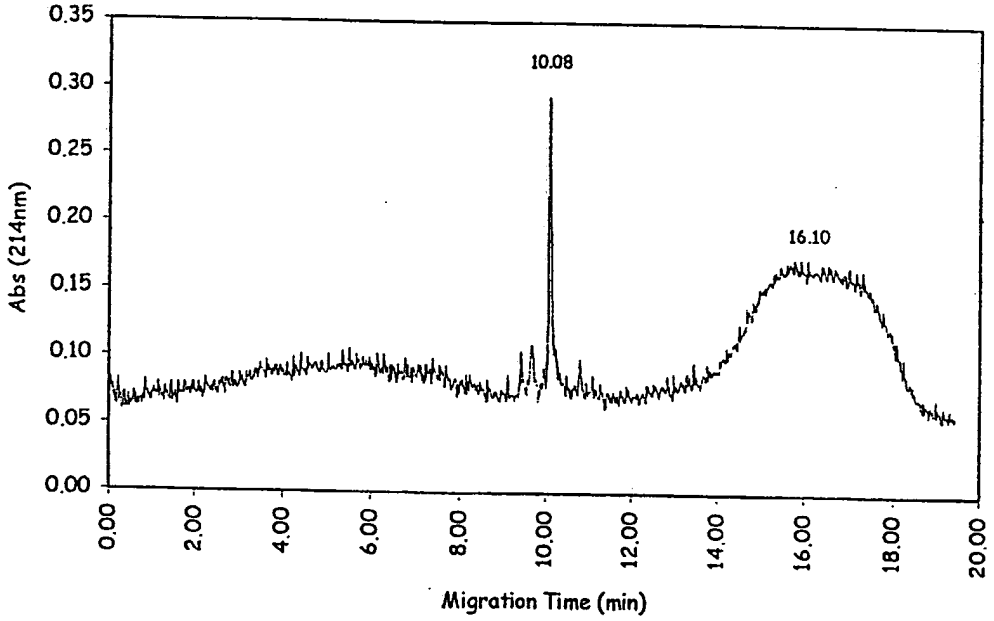

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Abs (214nm)
10.08
16.10
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
Migration Time (min)

FIG. 1

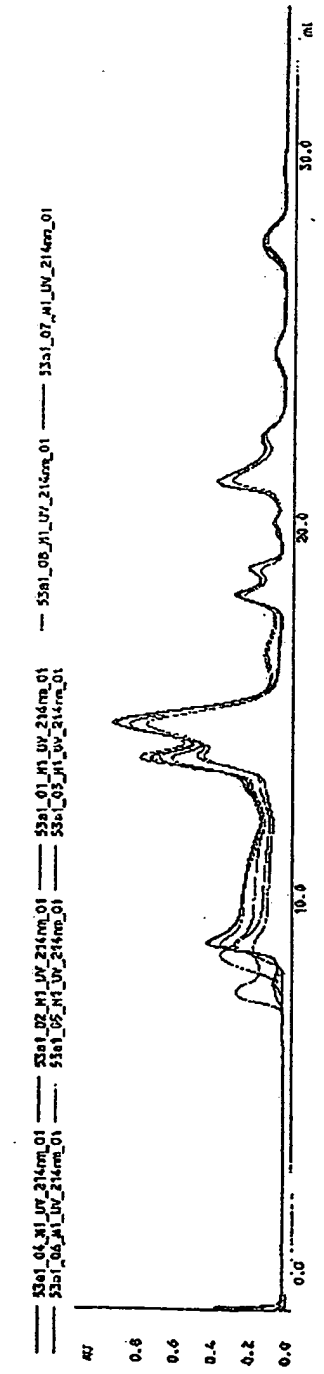


FIG. 2A

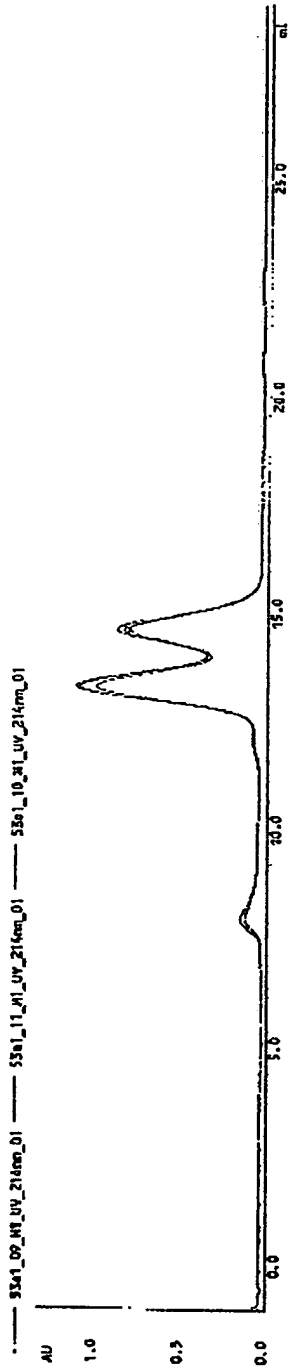


FIG. 2B

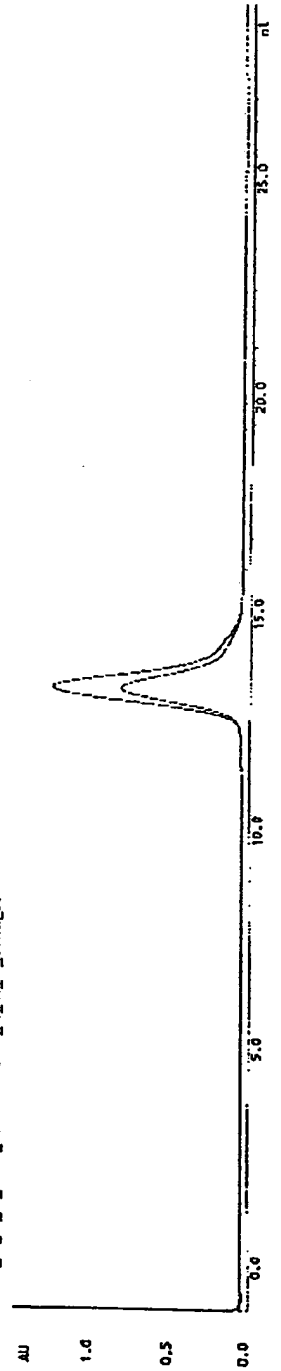


FIG. 2C

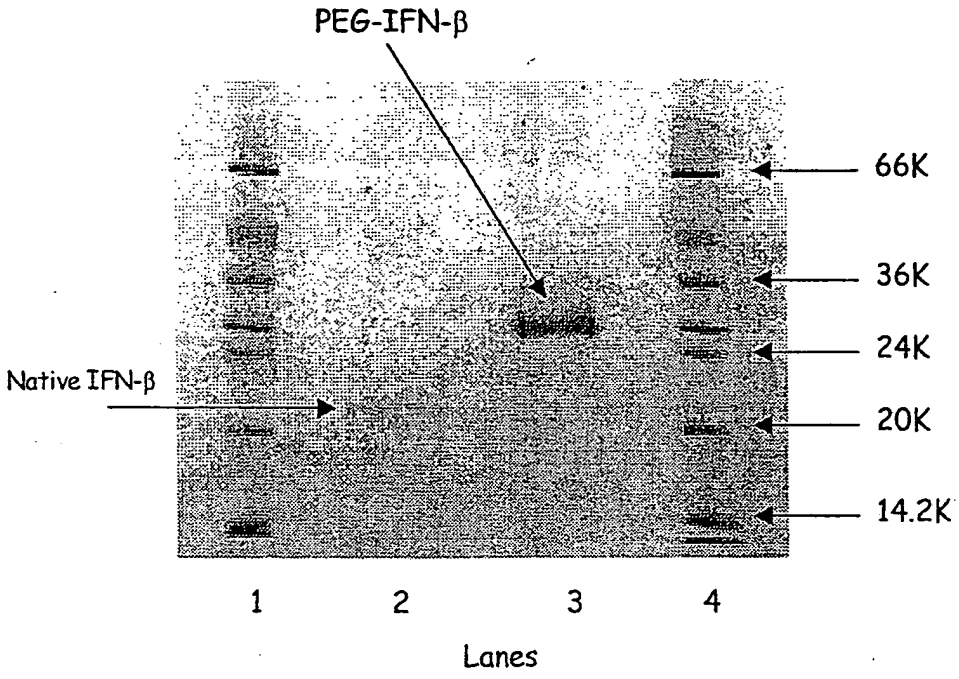

PEG-IFN-β
Native IFN-β
66K
36K
24K
20K
14.2K
1
2
3
4
Lanes

FIG. 3

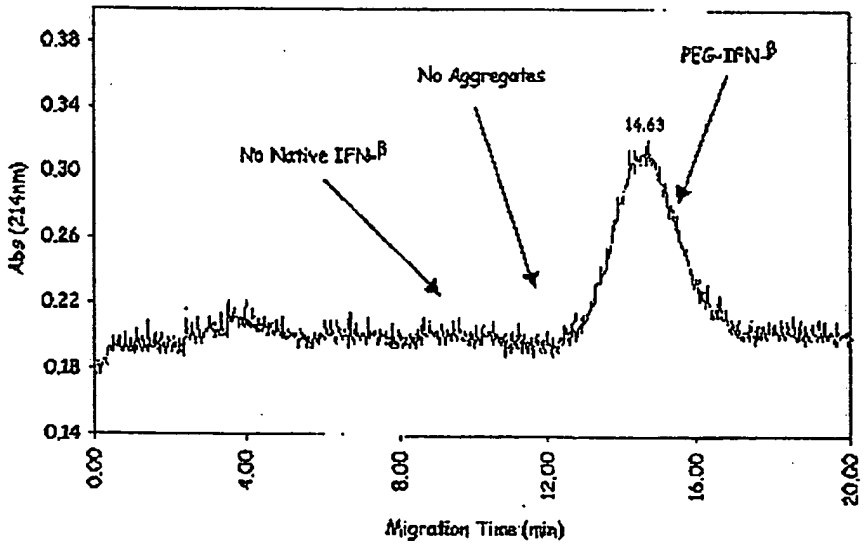

0.38
0.34
0.30
0.26
0.22
0.18
0.14
Abs (214nm)
No Native IFN-β
No Aggregates
14.63
PEG-IFN-β
0.00
4.00
8.00
12.00
16.00
20.00
Migration Time (min)

FIG. 4

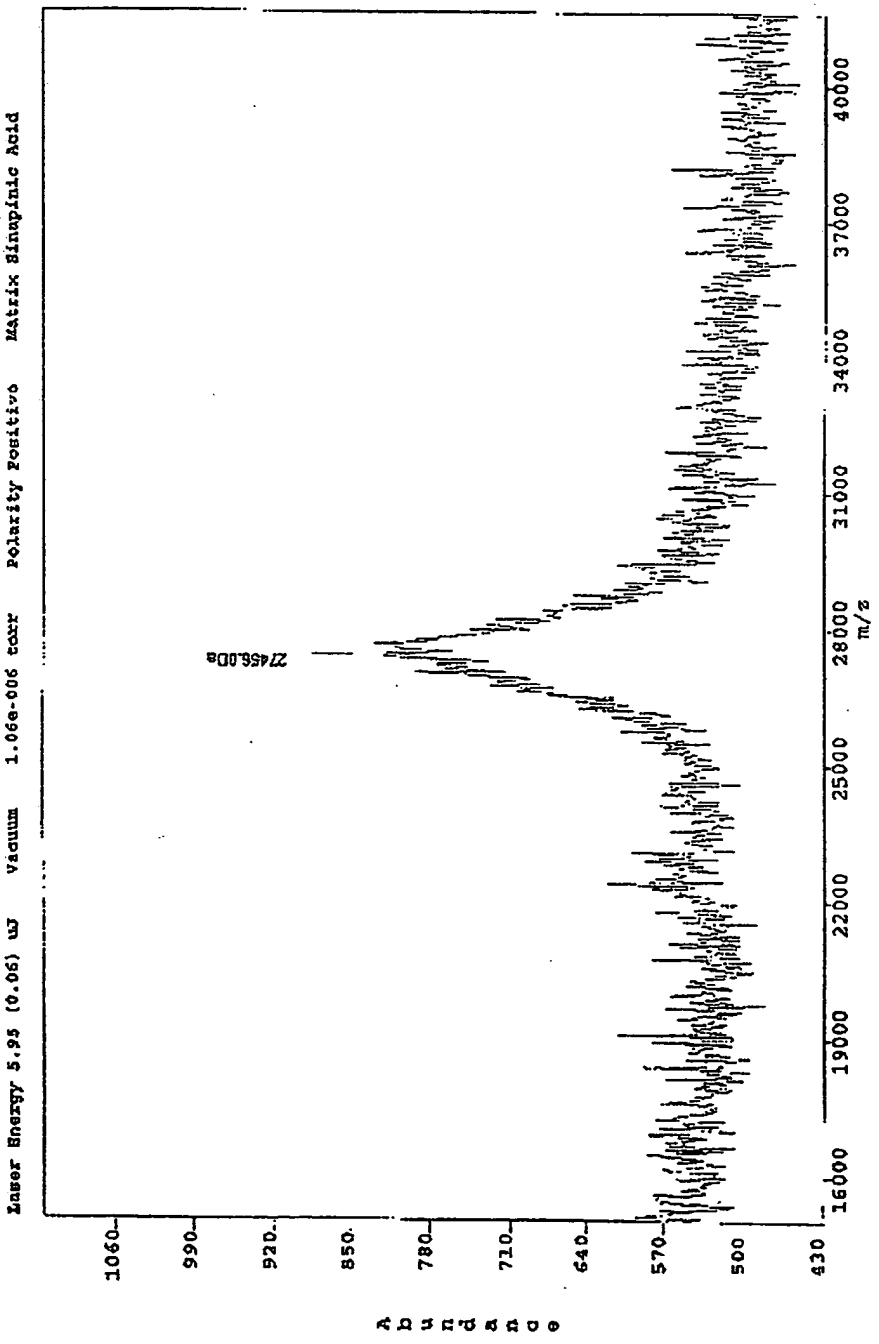

Laser Energy 5.95 (0.06) uJ Vacuum 1.06e-006 torr Polarity Positive Matrix Sinapinic Acid
27456.00Da
Abundance
m/z
1060
990
920
850
780
710
640
570
500
430
16000
19000
22000
25000
28000
31000
34000
37000
40000

FIG. 5

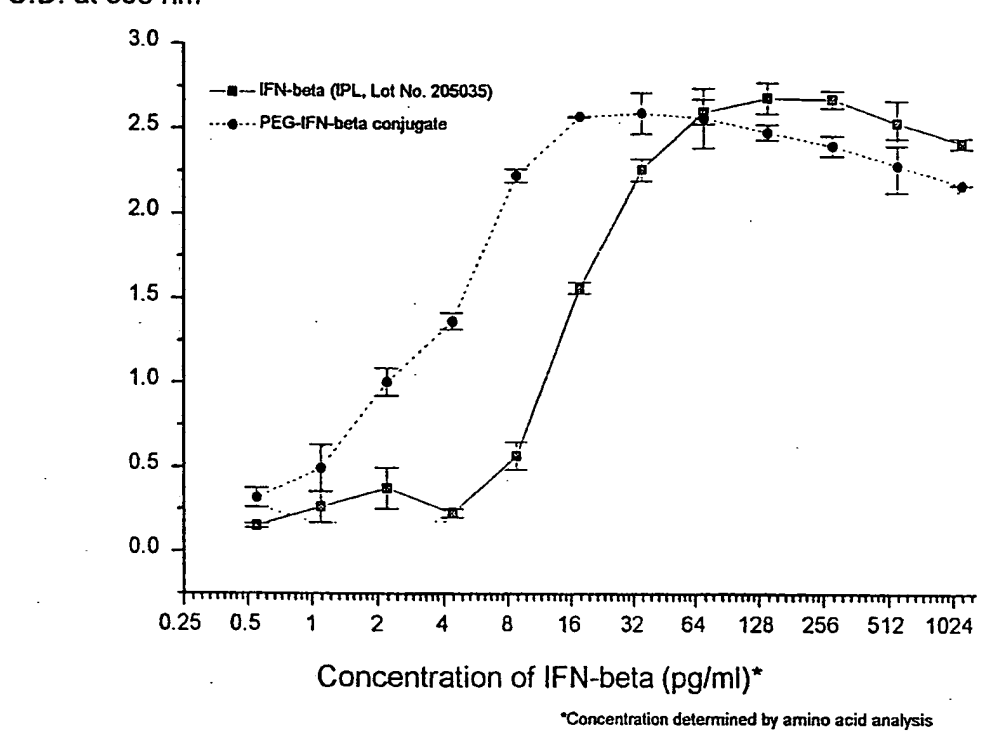

O.D. at 595 nm
3.0
2.5
2.0
1.5
1.0
0.5
0.0
IFN-beta (IPL, Lot No. 205035)
PEG-IFN-beta conjugate
0.25
0.5
1
2
4
8
16
32
64
128
256
512
1024
Concentration of IFN-beta (pg/ml)*
*Concentration determined by amino acid analysis

FIG. 6

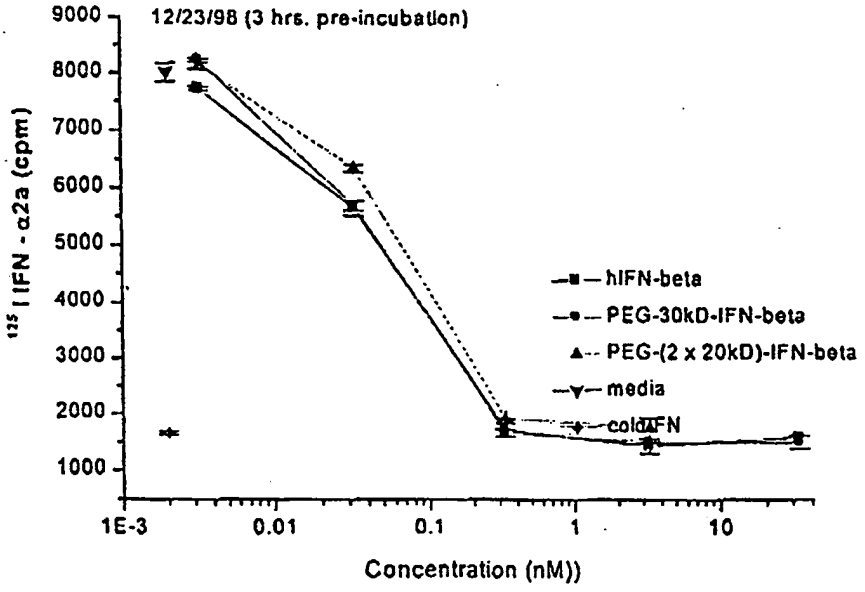

12/23/98 (3 hrs. pre-incubation)
125 I IFN - α2a (cpm)
9000
8000
7000
6000
5000
4000
3000
2000
1000
1E-3
0.01
0.1
1
10
Concentration (nM))
hIFN-beta
PEG-30kD-IFN-beta
PEG-(2 x 20kD)-IFN-beta
media
coldIFN

FIG. 7

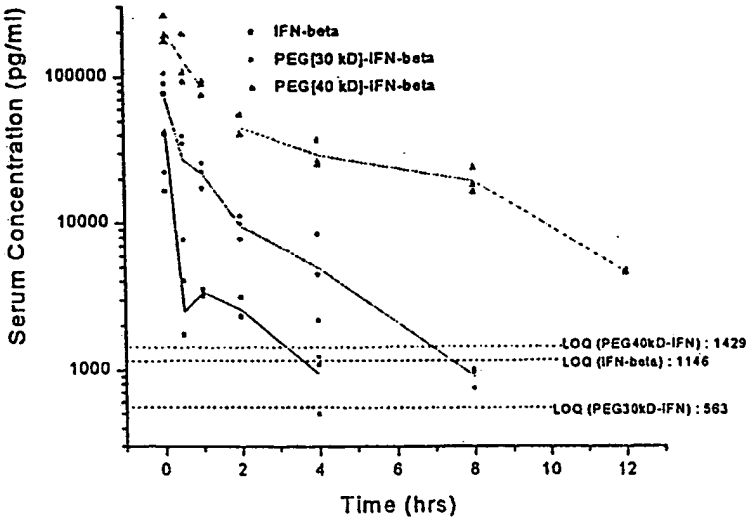

IFN-beta
PEG[30 kD]-IFN-beta
PEG[40 kD]-IFN-beta
Serum Concentration (pg/ml)
100000
10000
1000
LOQ (PEG40kD-IFN) : 1429
LOQ (IFN-beta) : 1146
LOQ (PEG30kD-IFN) : 563
0
2
4
6
8
10
12
Time (hrs)

FIG. 8

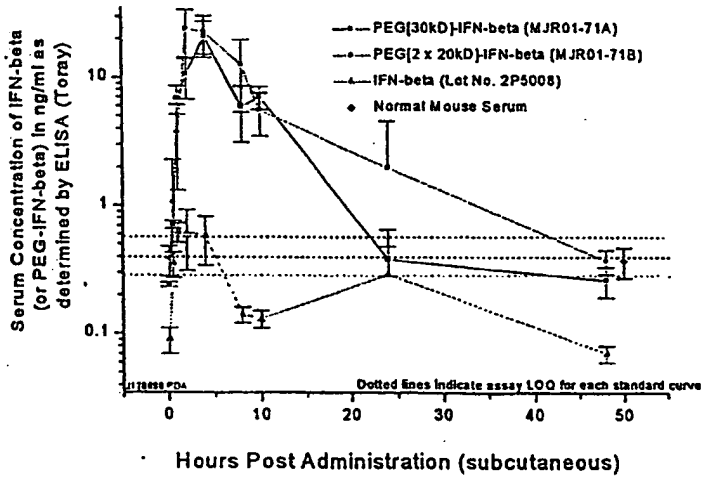

PEG[30kD]-IFN-beta (MJR01-71A)
PEG[2 x 20kD]-IFN-beta (MJR01-71B)
IFN-beta (Lot No. 2P5008)
Normal Mouse Serum
Serum Concentration of IFN-beta (or PEG-IFN-beta) in ng/ml as determined by ELISA (Toray)
10
1
0.1
Dotted lines indicate assay LOQ for each standard curve
0
10
20
30
40
50
Hours Post Administration (subcutaneous)

FIG. 9

ACS SYMPOSIUM SERIES **680**

Poly(ethylene glycol)

Chemistry and Biological Applications

J. Milton Harris, EDITOR
The University of Alabama at Huntsville

Samuel Zalipsky, EDITOR
SEQUUS Pharmaceuticals, Inc.

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Chapter 21

Hydrazide Derivatives of Poly(ethylene glycol) and Their Bioconjugates

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Hydrazide derivatives of poly(ethylene glycol) (PEG-Hz) have a number of attributes making them useful for preparation of conjugates, particularly of polypeptides and glycoproteins. They form conjugates in mildly acidic aqueous solutions via two modes of reactivity. The first one involves hydrazone formation with reactive carbonyls generated on the substrate molecule by several different methods. These include oxidation of oligosaccharide residues of glycoproteins, glyoxylate / Cu^{2+} -mediated transamination of the N-terminal residue of polypeptides, periodate oxidation of N-terminal Ser or Thr residues. The second mode involves coupling with carbodiimide-activated carboxyl groups forming diacylhydrazide linkages with PEG. Synthesis of PEG-Hz is straightforward by hydrazinolysis of esters of either carboxymethylated PEG or urethane-linked amino acid. Having an unusual amino acid, e.g. β-Ala, as part of the linker offers a convenient way for composition determination of protein conjugates, particularly those containing multiple chains of mPEG-O(C=O)-β-Ala-Hz, by amino acid analysis. Our work involving PEG-Hz conjugation, including examples of preparation of N-terminally modified polypeptides, oligosaccharide-linked glycoproteins, polypeptides modified on their carboxyl groups, and immunoconjugates of enzymes and liposomes is discussed in this review.

There are a number of reasons why polymers are conjugated to biologically-relevant molecules. In the most general sense, polymers are very effective modifiers of physicochemical properties such as molecular size, solubility, permeability, diffusion

rate. To achieve significant alteration of properties, macromolecular substrates , e.g. proteins, are often modified by multiple attachments of polymer chains, while in the case of low molecular weight molecules, one polymer chain of an appropriate length can often suffice. Among the polymers used for covalent modification of biologicals, PEG* is one of the most widely used. This is mainly due to the array of useful properties that PEG possesses. These include a wide range of solubilities in both aqueous and organic solutions, lack of toxicity and immunogenicity, non-biodegradability yet ease of excretion from living organisms. Conveyance of some of these properties onto PEG conjugates is often the principal motivation for their development (*1*). Another reason for extensive use of PEG lies in its availability in the form of well defined molecular weight materials of low dispersity. PEGs are usually available as bis-hydroxyls (HO-PEG-OH) or as monohydroxyls (CH_3O-PEG-OH, mPEG-OH). The art of transformation of these functionalities into a wide array of useful reactive groups suitable for bioconjugation reactions was perfected over the last two decades (*2*).

Most of the known methods for preparation of PEG conjugates rely on modification of amino groups of biologically active molecules with PEG-based electrophiles, usually an active ester or a reactive alkylating group (*1,3*). In the case of multifunctional biological molecules, e.g. polypeptides and proteins, other functional residues sometimes are desirable targets for modification with PEG. There is a great deal of interest in the development of site-specific reagents for protein conjugation. Hydrazides are attractive derivatives in relation to the latter two aspects of bioconjugation. The versatility of hydrazide functionality has been long known in the field of bioconjugates [see reviews (*4-6*)]. Hydrazides in general are good nucleophiles, yet relatively weak bases ($pK_a \approx 3$). Hence they are prone to many similar reactions that amino groups are known to undergo. Yet under mildly acidic conditions maintained in aqueous buffers, hydrazides are often more reactive than amines. There are two principal modes of reactivity of hydrazides that we were interested in, both of which are highly selective and useful for conjugation with peptides, proteins or glycoproteins (Scheme I). (A) Hydrazides react with carbonyls, which as will be discussed below, can be introduced onto various peptide ligands often in a site-specific manner. This reaction results in relatively stable hydrazone linkages that usually, in contrast to Schiff base analogs, do not require further stabilization by reduction. (B) Carboxyl groups of proteins, which are readily activated with water-soluble carbodiimide (e.g. EDC) at mildly acidic pH, react readily with hydrazides, while the amino groups on the same proteins remain deactivated due to protonation.

During the last decade we have used a number of hydrazide derivatives of PEG that were utilized in various conjugation protocols including protein modifications, preparation of immunoconjugates, and attachment of ligands to surfaces of PEG-grafted liposomes. While some of the methods developed were presented at various meetings

* See abbreviations list at the end of the chapter.

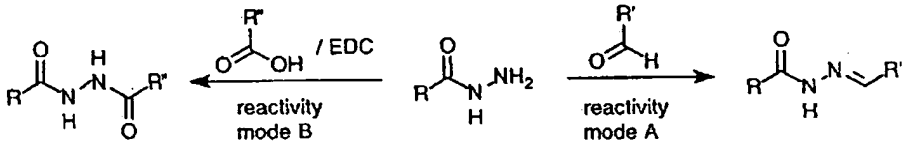
and were part of a few patent applications (*7-9*), only a few complete reports on this subject have been published (*10-14*). It is the purpose of this paper to summarize and to review our results with hydrazide derivatives of PEG and their bioconjugates with some emphasis on the previously unpublished aspects of this effort.

Results and Discussion

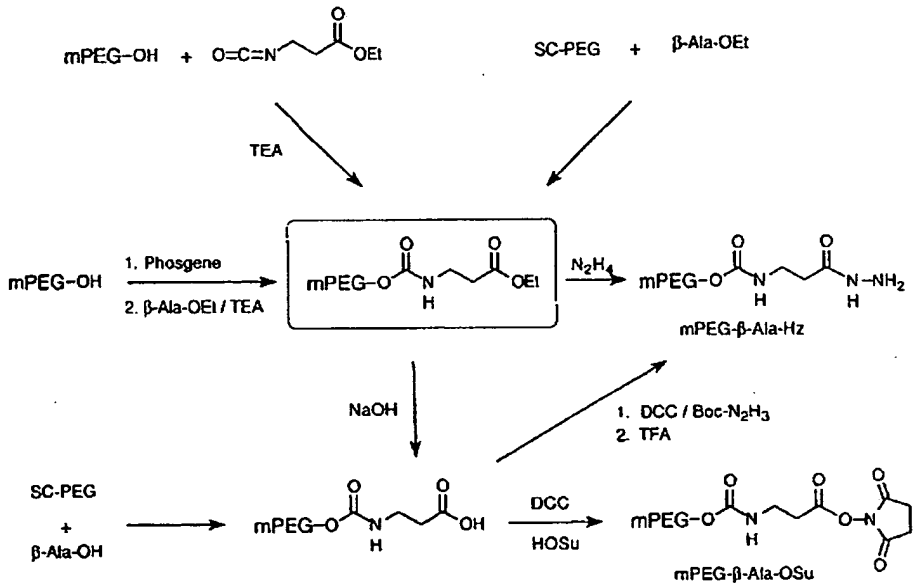
Background. The earlier literature made mention of hydrazide derivatives of PEG (PEG-Hz) only in a few places (*15,16*). Andresz *et al.* used a hydrazide derivative of PEG for preparation of mono- and oligo-saccharide conjugates interlinked through hydrazone linkages (*15*). The synthesis of PEG-Hz was accomplished in high yield by hydrazinolysis of esters of carboxymethylated-PEG. The same process was recently suggested as a convenient approach to PEG-hydrazides (*17*). The carboxymethyl hydrazides of PEG were mentioned in a few patents (*18-20*). In these cases, by a reaction with nitrous acid, the hydrazides were converted *in situ* into acyl azides and then used to acylate amino-containing residues of proteins. Reaction of mPEG-tresylate with an excess of adipic dihydrazide (ADH) was also utilized for introduction of hydrazido groups. The resulting product was used for conjugation with periodate oxidized ATP (*16*). The very low content of ATP in the final conjugate (0.06 mole ATP / mole PEG) indicates that this functionalization and conjugation approach was not efficient. Note that the tresylate chemistry and its use in bioconjugation protocols has been subjected to a serious revision (*21,22*), which makes the latter approach to PEG-hydrazide even more dubious.

Synthesis of PEG-Hz derivatives. Our approach to hydrazide derivatives utilized PEG-linked amino acid esters as starting materials. These materials are readily attainable by a few different pathways shown in Scheme II. They undergo hydrazinolysis without any significant cleavage of the urethane linkage to yield PEG-amino acid-hydrazides. Direct endcapping of the terminal hydroxyl groups of PEG with commercially available ethyl isocyanato-acetate or -propionate derivatives provide for a convenient approach to PEG-linked amino acid esters. (*10,23,24*). Alternatively PEG-chloroformate, easily generated by treatment of the polymer with phosgene, reacts with amino acid esters in high yield (*7*). Succinimidyl carbonate (*25*) or other active carbonates of PEG are also suitable for urethane linking amino acid residues to the polymer under mild conditions (*26,27*). The carboxyl group can be then readily converted into the hydrazide, for example by coupling *tert*-butyl carbazate followed by acidolytic removal of the Boc protecting group (*10,28*).

Most of the transformations shown in Scheme II are clean and high yielding reactions, easily amenable to scale up. Although all the reactions shown are for the β-



Scheme I. Two reactivity modes of hydrazides discussed in this review



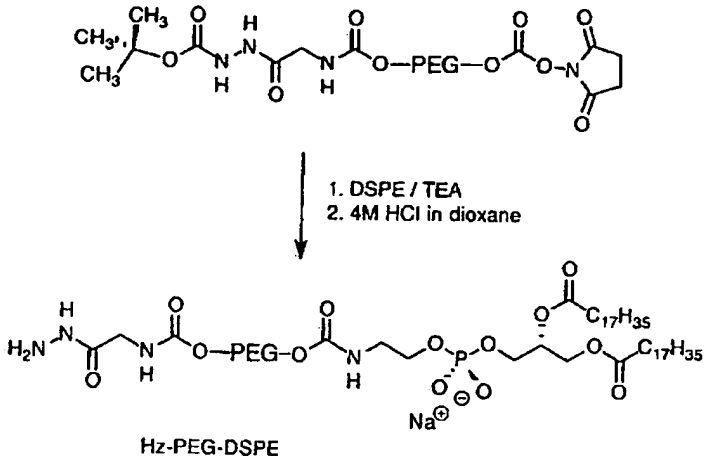
Scheme II. Synthesis of mPEG-β-Ala hydrazide and succinimidyl ester derivatives.

alanine derivatives, we have had equal success with glycine and some limited yet positive experience with other amino acid derivatives. Simpler Hz derivatives, PEG-carboxymethyl-Hz and PEG-carbazate, were synthesized by hydrazinolysis of the appropriate ethyl esters (*29*) or active carbonates (*25*) respectively.

Using α-hydroxy-ω-carboxy-PEG as a starting material, we prepared a new derivative containing the Boc-protected Hz-group and the amino reactive succinimidyl carbonate at the opposite terminals of the PEG chains (Scheme III). This heterobifunctional polymer was urethane-linked to DSPE, followed by acidolytic exposure of the Hz group (*10*). The resulting Hz-PEG-DSPE was used in preparation of PEG-grafted liposomes containing bioconjugation-prone Hz groups at the extremities of the polymer chains (*11-14,30*).

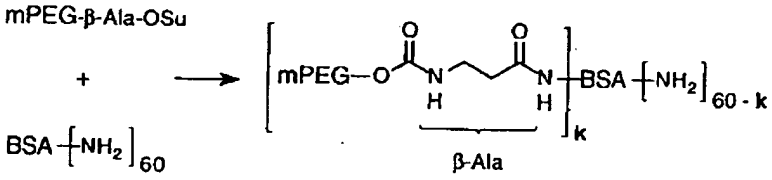
Amino acid as part of the linker: analytical implications. There are two principal reasons for choosing urethane-linked amino acid hydrazides. The ease of preparation is one of them. The second reason relates to the use of the amino acid linker as a marker for characterization of conjugates. Although the urethane linkage between PEG and an amino acid residue is stable to all the conditions to which the biological conjugate may be exposed (*31*), which includes *in vivo* or bioreactor applications, it can be quantitatively cleaved under conditions used for acid hydrolysis of proteins as performed for amino acid analysis (usually 6 N HCl, 110 °C, 24 h). Amino acid analysis (AAA), in general, can be used with high precision for protein quantitation. The positioning of an unusual amino acid that does not appear in natural polypeptide sequences, like β-Ala or Nle (*26*), as part of the linker between PEG and protein allows for convenient determination of the composition of such conjugates by AAA. In fact, knowing the amino acid composition of a protein, it is possible to determine both concentration and composition of its PEG conjugate in just one AAA run consuming only a small aliquot of the conjugate preparation, < 50 μg. This is particularly attractive for exploratory work when limited quantities of the protein are available for modification.

The traditional method for characterization of amino group-linked PEG-proteins (*32*) involved determination of the percentage of unreacted amino groups on the modified protein with 2,4,6-trinitrobenzenesulfonate (TNBS) (*33*). With all its shortcomings this method was widely used, and is still in use, in conjunction with a variety of PEG-based amino-reactive reagents (*3*). Note that for both modes of protein or glycoprotein modifications with hydrazide reagents, that we were interested in, there is no comparable analytical methodology for determination of reacted sites. As an initial validation exercise we decided to compare the results of composition analysis performed by both amino group determination and AAA on a series of BSA conjugates obtained using the succinimidyl ester of urethane-linked mPEG-β-Ala (see Scheme II). The results of these analyses, calculated in terms of the number of mPEG residues per BSA molecule, are summarized in Table I. We compared both fluorescamine (*34*) and



Scheme III. Preparation of Hz-PEG-DSPE (*10*).

Table I. Determination of compositions (k values) of $[mPEG]_k$-BSA conjugates, prepared as shown below, by amino acid analysis and by amino group quantitation.



Sample	β-Ala[a]	TNBS[b]	Fluorescamine[c]
1	44.4	42.6	37.6
2	28.3	29.8	26.6
3	18.0	19.7	13.3
4	12.0	10.4	9.5
5	6.2	5.5	3.4

[a] Determined by AAA of the hydrolysates.

[b] Determined by TNBS assay according to Habceb (*33*).

[c] Determined by Fluorescamine assay according to Stocks *et al.* (*34*).

TNBS methods (*33*) which determine the fraction of unreacted amino groups on the modified protein. The results of the latter method were consistently in closer agreement with AAA through the entire range of the extent of BSA modification. These data were consistent with our previous observations that succinimidyl esters of PEG are selective in their reactivity toward amines (*25*). Further the TNBS procedure of Habeeb (*33*) results in a more accurate determination of amino groups on proteins. Note that the fluorescence method consistently underestimated the extent of modification, probably due to the incomplete reaction of fluorescamine with the buried amino groups of BSA, and also possibly because of quenching effects. Sartore *et al.* published results of similar measurements performed on a few mPEG-proteins modified to various degrees with the succinimidyl ester of urethane-linked mPEG-Nle (*26*). The AAA and TNBS results were often markedly different, with the latter method usually overestimating the extent of modification. The results summarized in Table I suggest two conclusions. (1) The composition of mPEG-β-Ala-protein can be accurately determined by quantitation of β-Ala by AAA. (2) Fluorescamine is not a very accurate method for determination of the extent of modification of proteins.

Based on the first conclusion, quantitation of β-Ala by AAA has proven to be quite useful in characterization of conjugates derived from mPEG-β-Ala-Hz. One of the most useful conjugation reactions of this reagent involves modification of oxidized glycoproteins. Oxidation of vicinal diols of the oligosacharide moiety with periodate was utilized for generation of reactive aldehyde groups, which were then converted into hydrazone attachments with mPEG-β-Ala-Hz (Scheme I, mode A). For determination of the optimal conditions for this conjugation reaction we used glucose oxidase (GO) as a model substrate. This enzyme is readily available and inexpensive. It is a glycoprotein containing 18% carbohydrate, which makes it possible to achieve a broad range of modifications. By performing conjugation reactions at various pH values, other than the standard conditions, a series of PEG-GO hydrazones was generated. The purified conjugates were analyzed by SEC-HPLC and also subjected to AAA for quantitation of the incorporated β-Ala residues. As illustrated in Figure 1 the conjugate obtained at pH 5.5 eluted with the shortest retention time on SEC-HPLC, indicating that this was the largest molecular size with the highest extent of modification. Accordingly, the largest number of mPEG-β-Ala residues was detected in this conjugate (Figure 1). Although this conjugation reaction generally can be performed in a relatively broad pH range, the best results are obtained at pH 5 - 6.5, provided the protein is not inactivated.

The two modes of hydrazide reactivity. Utilizing these conjugation conditions we prepared hydrazone conjugates of PEG-hydrazides and various glycoproteins (*7*) (see Table II). Immunoglobulins are in particular attractive as targets of this approach. These glycoproteins contain ≈ 4% carbohydrate located on the Fc region of the molecule far from the antigen binding sites (*6*). Therefore the site specificity is the main

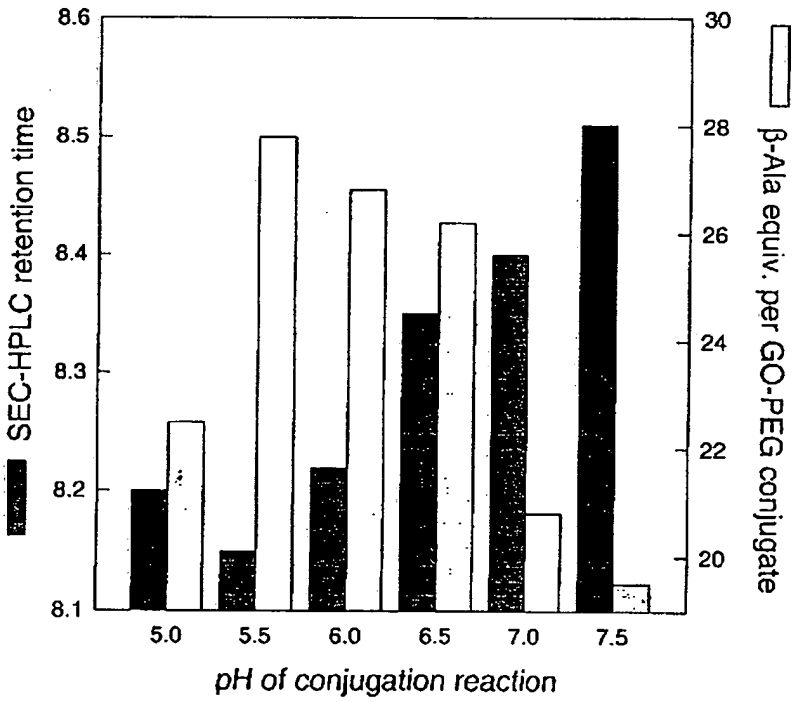
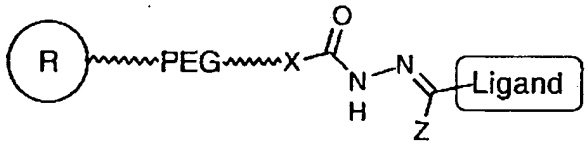


Figure 1. Relationship between the number of mPEG-β-Ala-Hz residues linked per glycoprotein molecule of glucose oxidase and pH of the conjugation reaction.
GO (3 mg/ml) was oxidized with 20 mM $NaIO_4$ for 1 h and then treated with ≈ 2000-fold molar excess of mPEG-5k-β-Ala-Hz. The purified conjugates obtained at various pH values, under otherwise identical conditions, were analyzed by SEC-HPLC and β-Ala content determined by AAA.

Table II. Summary of PEG-hydrazones discussed in this manuscript of general formula[a]



R	X	Ligand	Comments
CH_3O-	β-Ala deriv.	IgG	Periodate-oxidized IgG reacted with an excess mPEG-Hz, 2-9 mPEG chains / IgG.
CH_3O-	β-Ala deriv.	Glucose Oxidase	Used as a model glycoprotein to determine optimal conditions of conjugation (Fig. 1).
CH_3O-	β-Ala deriv.	Ovalbumin	Periodate-oxidized glycoprotein was modified as a model, 2-5 mPEG residues / ovalbumin.
CH_3O-	OCH_2 O NH	Hormone glycoprotein of 29 kDa	Site-specific PEGylation on N-terminal. Protein transaminated by glyoxalate / Cu^{2+}, and site-specifically coupled with Hz derivatives of mPEG-5k. Best yield was obtained with the carbazate derivative (X = O).
HRP-	Gly deriv.	IgG	HRP modified on its carboxyl groups with Hz-PEG-Hz, 3-4 residues / HRP. Periodate-treated IgG was labeled with HRP-PEG-Hz.
DSPE anchored in liposome bilayer	Gly or β-Ala derivs	IgG	Immunoliposomes were prepared by coupling various periodate-treated IgGs with Hz-PEG-liposomes. The number of IgG per vesicle can be controlled by changing conditions, e.g. ratio of the reactants.
DSPE anchored in liposome bilayer	Gly or β-Ala derivs	YIGSR-NH_2	Site-specific conjugation to N-terminal. TYIGSR-NH_2 was converted into N-α-glyoxylyl-YIGSR-NH_2 with periodate and then coupled with Hz-PEG-liposomes. Ligand densities of hundreds per vesicle.

[a] Z is usually H or an alkyl residue of a reactive ketone.

advantage of this approach to modification of IgGs and other glycoproteins (*5*). Due to the usually low carbohydrate content, however, we found it difficult to attach multiple chains of PEG per IgG. Note that the generation of aldehyde groups on many glycoproteins can be achieved enzymatically by utilizing neuraminidase and galactose oxidase (*35*). These conditions are considerably milder than periodate treatment, which is known to affect several oxidation-prone amino acid residues (Trp, Tyr, sulfur-containing amino acids, N-terminal Ser and Thr). Occasionally we have encountered IgG that was inactivated by a periodate treatment, presumably by oxidation of one or more of these sensitive residues (*36*).

Oxidation of the oligosaccharide moiety of glycoproteins is only one of several useful methods to generate reactive carbonyls on polypeptides. In a more general sense polypeptides bearing reactive carbonyl(s) are suitable for conjugation with PEG-Hz derivatives via pathway A of Scheme I. For example, King *et al.* (*37*) described utilization of the succinimidyl ester of formyl benzoic acid as a reagent for introduction of aromatic aldehydes onto proteins by substituting some of the amino groups. Although this protein modification is random, the aryl aldehydes were shown to possess good reactivity toward hydrazides and excellent selectivity over amines.

A few very useful methods for the introduction of reactive carbonyls onto peptides, selectively on their N-terminal residues, were described by Dixon and Fields (*38,39*). The most general of these reactions, transamination, is glyoxylate mediated and catalyzed by bivalent metal ions, usually Cu^{2+}. The transamination reaction results in conversion of the N-terminal residue of the peptide into a α-keto acid residue. As will be illustrated by a specific example below, the transaminated polypeptide can react with hydrazide derivatives of mPEG producing well defined conjugates containing only one polymer chain linked to the N-terminus. Conjugation of transaminated interleukin-1 receptor antagonist to aminooxy-PEG derivatives via an oxime linkage was recently reported (*40*). The peptides containing N-terminal Ser or Thr residues undergo very rapid periodate oxidation (≈ 1000 fold faster than 1,2 diol) resulting in destruction of these amino acid residues and formation of N-α-glyoxylyl-peptides (*38,39*). This very mild approach to aldehyde-bearing peptides can be easily followed by hydrazone formation with PEG-hydrazide (*14*).

Covalent attachment of PEG-Hz to carbodiimide-activated carboxyl groups of proteins (Scheme I, mode B) proved useful in a number of cases (*7*). This approach is particularly attractive as an alternative to a more common amino group modification when the protein has very few amines or it is inactivated by their modification. This mode of reactivity of PEG-Hz results in a random modification of available Asp and Glu residues via diacyl hydrazide attachments of the polymer chains. Under conditions optimal for water-soluble carbodiimide reactivity, around pH 5 (*41*), the conjugation of proteins with mPEG-β-Ala-Hz proceeds cleanly without any detectable modification of the amino groups (see the experimental procedure for mPEG-GCSF preparation). As

would be expected, and judging from the isoelectric focusing analysis, the product of such a protein modification consists of multiple components with higher pI values than the parent protein. While using this mode of hydrazide reactivity, one has to be aware of carbodiimide-associated side reactions (*42*), and try to suppress or minimize them depending on their adverse effect on the final conjugate. Among the known protein modification reactions of carbodiimides are formation of O-isourea and S-isourea with Tyr and Cys residues respectively, and particularly troublesome side reaction of N-acylurea formation with Asp and Glu derived carboxyls (*42*). Consistent with our own observations, suppression of N-acylurea formation by carbodiimide / hydrazide combination was reported under conditions that predominantly yield this side-product with carbodiimide / amine (*43*).

Specific examples of PEG-Hz conjugates. Wilchek and coworkers described preparation of the Hz-containing enzymes (horseradish peroxidase and alkaline phosphatase) which were used for labeling glycoproteins (*44*). In analogy with this strategy, we reacted a large excess of dihydrazide of PEG-4k with HRP, utilizing the carboxyl groups of the enzyme for the attachment of the homobifunctional PEG (Scheme I, B). The HRP-PEG-Hz, produced in this reaction contained 3-4 PEG-Hz chains per molecule and retained over 90% of the specific activity of the parent enzyme, as was determined using o-phenylenediamine as the substrate. The Hz-groups on the far ends of the HRP adduct were utilized for conjugation with periodate-oxidized IgG (α-HCG) utilizing the specific reactivity of its oligosaccharide moiety (Table II). Despite the presence of several PEG chains on the surface of the enzyme, and in contrast to some previously reported observations (*45*), the HRP-PEG-Hz conjugate bound readily, just like the parent HRP, to an affinity column of Concanavalin A. We took advantage of this binding for purification of the HRP-PEG-Hz conjugate from the excess of the PEG-Hz reagent and also for purification of the final HRP-PEG-IgG product.

N-terminal protein PEGylation. A glycoprotein hormone of 29 kDa (apparent molecular weight 35 kDa by SDS-PAGE) was site-specifically PEGylated on its N-terminal in two steps (see Table II). The protein was first transaminated according to Dixon and Fields (*38*) and then covalently modified on the freshly exposed α-keto acyl residue with either mPEG-hydrazide or mPEG-carbazate derivatives of 5 kDa. A single conjugate of 34 kDa (46 kDa by SDS-PAGE) was formed in both cases in 25 and 95 % yield, respectively. The conjugation reaction was enhanced by the presence of SDS in the reaction mixture, probably by partial unfolding of the protein and enhanced exposure of the N-terminal residue. The mPEG-glycoprotein was characterized by electrophoresis methods on 4-15% SDS-PAGE gradient gels. Figure 2 shows a Coomassie stained gel of the native, transaminated, and mPEG-5k-conjugated derivatives of the glycoprotein. Figure 3 shows the Western blot experiment with the

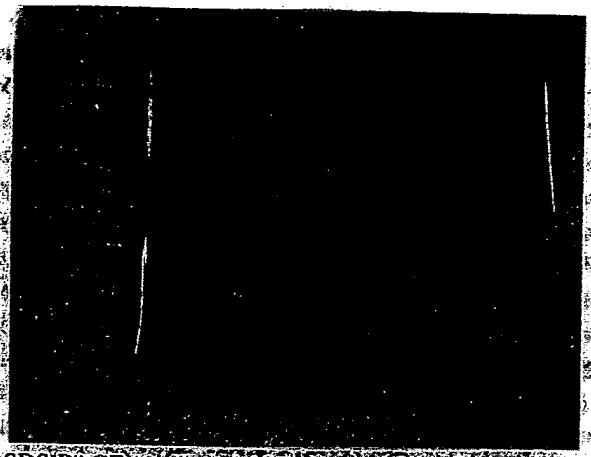


Figure 2. SDS-PAGE (gradient 4-15%, Coomassie blue staining) of a glycoprotein hormone and its N-terminal conjugate with mPEG-5k. Lane 1: molecular weight markers from top to bottom: myosin (200 kDa), phosphorylase B (97.4 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa). Lane 2: native glycoprotein. Lane 3: transaminated glycoprotein. Lane 4: glycoprotein modified with mPEG-carbazate; lane 5, glycoprotein modified with mPEG-OCH_2-Hz.

Figure 3. Western blot analysis of glycoprotein hormone and its N-terminal conjugate with mPEG-5k. The same lane assignments as in Fig. 2. The following prestained molecular weight markers (and their apparent molecular weights) were used (lane 1): Phosphorylase B (106 kDa), bovine serum albumin (80 kDa), ovalbumin (49.5 kDa), trypsin inhibitor (32.5 kDa), lysozyme (18.5 kDa). Electrophoresis was performed on 4-15% gradient gels. Proteins were electrophoretically transferred to nitrocellulose membrane. The membrane was first incubated with the primary antibody recognizing the glycoprotein, and then with a secondary antibody-AP conjugate recognizing the Fc portion of the primary antibody. AP color developing substrate was used for visualization.

same lane assignments for the samples as in Figure 2. Both figures show that the molecular weight of the transaminated protein in lane 3 is essentially the same as that of the native protein. Disapearance of one Ala residue on the transaminated derivative was independently confirmed. Upon PEG conjugation with the mPEG-Hz linkers (lanes 4 and 5) the molecular weight of the PEG-conjugate is shifted to a higher molecular weight. The results obtained from the gel electrophoresis and Western blot experiments demonstrated that PEG-conjugation was complete after transamination, giving rise to a unimolecular mPEG-hydrazone conjugate. The detection of only one molecular-weight species of the conjugate demonstrates the specificity of the reaction. These observations were further corroborated by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The mPEG-5k-carbazate yielded an experimental molecular mass of 5157 as shown in Figure 4A. For the native glycoprotein in Figure 4B the peak labelled at m/z 28570 represents the singly charged monomer. For the mPEG-5k-glycoprotein shown in Figure 4C the peak for the singly charged monomer is seen at m/z 34280, confirming the addition of one mPEG chain of 5 kDa onto the glycoprotein. The stability of the mPEG-carbazate-glycoprotein was probed by incubating the conjugate in acetate buffer (0.1 M, pH 4.5) over a period of 4 days. No breakdown or degradation was observed with SDS-PAGE despite the acidic conditions. Although the *in vitro* activity of this particular conjugate was decreased, the *in vivo* circulating time and hence the bioavailability was enhanced.

Note that the issue of composition determination by AAA is more relevant to conjugates composed of multiple mPEG-chains. For the conjugates containing only one polymer chain per protein, as discussed above for the N-terminal modification, it is sufficient to demonstrate completion of the reaction and consumption of the starting materials. In these cases there is little advantage to having a reference amino acid as part of the linker, and simpler derivatives like the hydrazide of carboxymethylated PEG (*15,17*) or PEG-carbazate are sufficient. It is intriguing, that the latter derivative was the most effective PEGylating reagent in the last example.

Ligand-bearing PEG-grafted liposomes. Long-circulating, RES-evading liposomes possess these valuable properties due to external surface-grafted PEG chains, usually of 1-5 kDa (*46*). We found that recently-introduced Hz-PEG-DSPE (Scheme III) can completely replace the mPEG-DSPE conjugate, originally used for preparation of such liposomes, without any measurable effect on the pharmacokinetic behavior (Figure 5) or tissue distribution. However, in contrast to the inert methoxy end groups of mPEG, the Hz-terminated polymer chains find use for attachment of various biologically relevant ligands. The most obvious application of such systems is in targeted delivery of liposomal drugs, and IgG is most commonly used as the targeting moiety. A variety of immunoglobulins were conjugated to Hz-PEG-2k-liposomes through their carbohydrate residues, taking advantage of the previously developed

methods for coupling mPEG-Hz (Table II). The antigen binding activity of the immunoliposomes prepared by this methodology was usually well preserved, although the pharmacokinetic behaviour typically required some fine-tuning to balance the beneficial properties of the liposomes and to optimize the density of the attached IgG residues (*11,30,36,47*). As illustrated in Figure 5 immunoliposomes containing on average 10 IgG residues per lipid vesicle exhibited prolonged systemic circulation although their clearance rate was noticeably faster than the parent liposomes and the free IgG (*11*). Note that both ^{125}I and ^{67}Ga labels placed on the protein and liposome, respectively, exhibited the same pharmacokinetic curves, indicating that the components of the immunoliposome do not dissociate while in blood circulation.

Another important application of ligand-bearing, long-circulating liposomes pertains to their use as platforms for presentation of various small ligands. Such low molecular weight molecules (peptides, oligosaccharides, etc.) in their free form are promptly cleared from the systemic circulation. We devised a convenient method for attaching peptides to the Hz-extremities of the liposome-grafted PEG chains. Peptides containing Ser or Thr residues at their N-termini can be readily obtained by modern peptide synthesis methods. Brief treatment of such peptides with periodate cleaves the 1-amino-2-hydroxyl residue resulting in N-α-glyoxylyl peptide, which reacts with Hz-PEG-liposomes producing hydrazone attachments. The conjugation is site-specific to the N-terminal residue of the peptide. It is performed in a one-pot procedure, and requires only one chromatography or dialysis step for the final product purification (*14*). We demonstrated the feasibility of this approach to peptidoliposomes with the cell adhesive peptide sequence, TYIGSR-NH_2. It was possible to attach several hundreds of the peptide residues per liposome (100 nm) without any difficulties. Double-label pharmacokinetic experiments performed in rats with peptidoliposomes (200 peptide residues per lipid vesicle), containing ^{125}I-YIGSR-NH_2 and ^{67}Ga-desferal loaded in the internal aqueous compartment, showed that both labels exhibited identical clearance characteristics. Thus the peptide and the liposome remained conjugated in the blood plasma and remained there for only slightly shorter periods of time than the parent Hz-PEG-liposomes. This indicates that the hydrazone attachments formed by liposome-grafted Hz-PEG and N-α-glyoxylyl-YIGSR-NH_2 were stable under the conditions existing *in vivo* (*14*). These experiments suggested that this general approach might be useful for increasing the systemic exposure of biologically active short peptide sequences.

Concluding Remarks

As illustrated by the examples above, hydrazide derivatives of PEG are useful for preparation of peptide and protein adducts via a number of conjugation methods. Mild conjugation conditions, high selectivity, and site-specificity of the coupling reactions are among the beneficial characteristics of these methods. The latter feature is of particularly

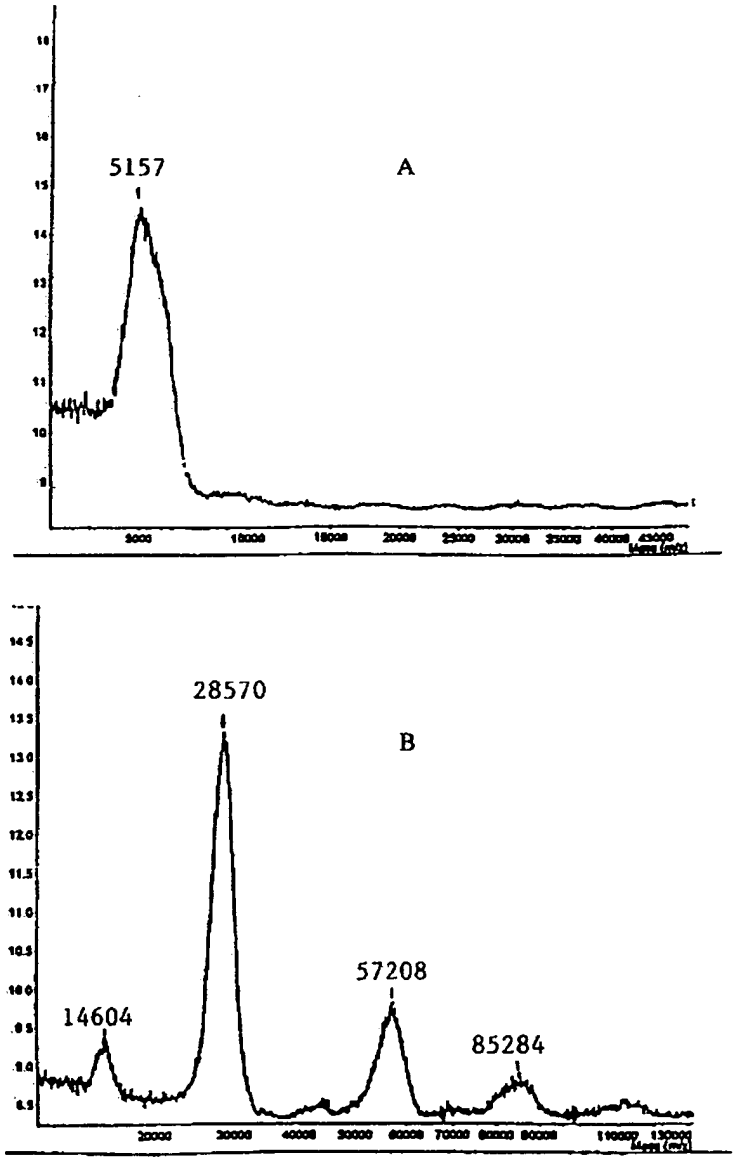


Figure 4. MALDI-MS of an N-terminally PEGylated glycoprotein hormone and the individual components of the conjugate. (A) mPEG-carbazate, m/z 5157; (B) Singly charged monomer of the native glycoprotein, m/z 28570. The signals at m/z 57208 and 85284 represent the dimer and trimer. Doubly charged ion, m/z 14604. (C) Singly charged monomer of mPEG-glycoprotein hydrazone, m/z 34280. The signals at m/z 69071 and 102955 represent the dimer and trimer. Doubly charged ion, m/z 17092. The spectra were acquired on a Finnigan-MAT time of flight LaserMAT 2000 mass spectrometer.

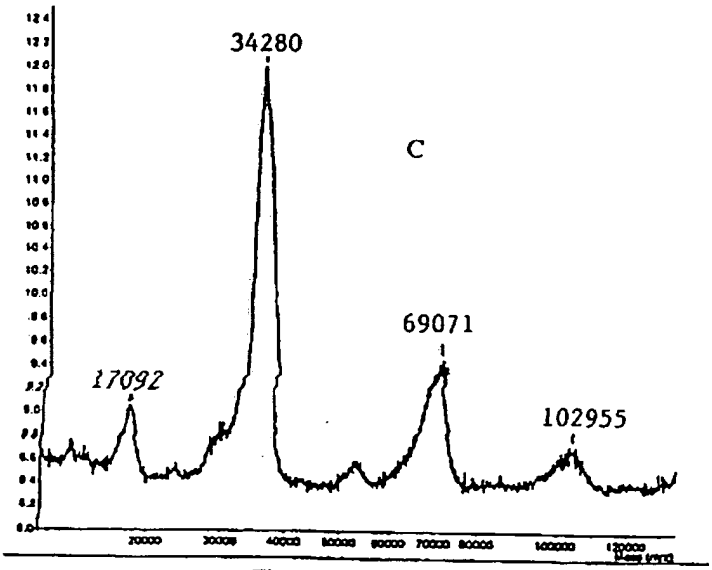


Figure 4. *Continued.*

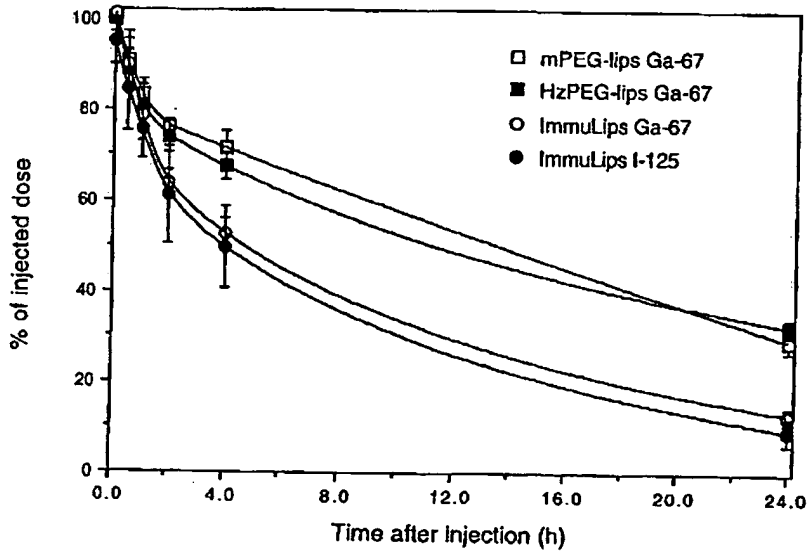


Figure 5. Blood lifetimes of liposomes prepared from hydrogenated soy phosphatidyl choline, cholesterol, mPEG-DSPE (mole ratio 1.85:1:0.15), same formulation containing Hz-PEG-DSPE (5 mole %) instead of the mPEG analog, and immunoliposomes prepared by coupling oxidized IgG to the Hz-PEG-liposomes. The immunoliposomes were labeled with ^{125}I (protein label) and ^{67}Ga-desferal in the internal aqueous compartment. Four Sprague-Dawrey rats were injected with each liposomal formulation via a tail vein. Samples collected at various times by retro-orbital bleeding were used to determine radioactivity in blood.

great utility for preparation of N-terminally modified polypeptides or glycoproteins carrying PEG chains only on their oligosaccharide residues. For both modes of reactivity discussed here, hydrazone formation with reactive carbonyls or modification of carboxyl groups forming diacylated hydrazine attachments, excellent selectivity against the amino groups was observed. Considering that most of the commonly used PEG-based reagents are designed for modification of amino groups in a rather random fashion, addition of the PEG-hydrazides to the arsenal of polypeptide reacting agents markedly expands the bioconjugate design options. Since in many instances optimal preservation of biological activity of the PEGylated polypeptides is dependent on the total number and the positioning of the polymer chain attachment sites (*1,3*), use of site-specific methods for conjugate preparation is likely to yield well defined adducts with better preservation of biological activity. It can be concluded with confidence that many more useful bioconjugates will be constructed utilizing the chemical versatility of PEG-hydrazides.

Experimantal Procedures

TNBS determination of Hz groups. TNBS assay as described for determination of amino groups (*48*) was used with the following modifications. ADH purified by recrystalization was used to prepare standard solutions with concentrations in the range 0 - 0.1 mequiv. Hz/l. Potassium borate (0.2 M, pH 9.3) was used for preparation of the standard and the sample solutions. A sample of Hz-containing material (1 ml solution, < 0.1 mequiv. Hz/l) in the borate buffer was treated with TNBS (25 µl, 30 mM). After ≈ 45 min incubation at 25 °C the maroon-red color was measured at 500 nm. A calibration curve was obtained with the standard ADH solutions. Typically ε_{500} = 16,500 - 17,000 $M^{-1} \cdot cm^{-1}$ was obtained by this procedure.

Test for presence of PEG in aqueous solutions (PMA test). The presence of PEG was detected by mixing a drop of a sample solution with a drop of PMA solution (1% in 1 N HCl). Immediate appearance of white precipitate of the interpolymer PEG-PMA complex indicated the presence of PEG. This test proved to be very specific to the polyether backbone of PEG, and was not influenced by presence of salts in the solution or derivatization / conjugation of PEG, as long as pH < 4 was maintained (*24,29,49*).

Preparation of mPEG-O(C=O)-β-Ala-OEt (7). mPEG (100 g, 20 mmol) was dissolved in toluene (250 ml) and azeotropically dried. The solution was brought to 25 °C, diluted with methylene chloride (50 ml) and then treated with phosgene (30 ml of 20% toluene solution, 56 mmol) overnight. The solvents and the excess of phosgene were removed by rotary evaporation under vacuum. The solid residue of polymeric

chloroformate was dissolved in methylene chloride (90 ml) and treated with β-Ala-OEt hydrochloride (6.1 g, 40 mmol) predissolved in methylene chloride (total vol. 30 ml) followed by TEA (8.4 ml, 60 mmol). Approximately 30 min later the solution was diluted with toluene (50 ml), filtered and evaporated to dryness. The crude product was dissolved in warm (50 °C) ethyl acetate (500 ml) and filtered through celite. The filtrate was diluted with *iso*-propanol to a total volume of 1000 ml and left overnight at 25 °C to facilitate precipitation of the product. Another recrystallization of the product from *iso*-propanol was performed. The yield of the dried mPEG-β-Ala-OEt was 98 g (95 %). I.R. (neat): 3341 (N-H), 1723 (C=O,urethane) cm^{-1}. ^{1}H-NMR ($CDCl_3$): δ 1.27 (t, J = 7 Hz, $C\underline{H}_3CH_2$, 3H), 2.53 (t, J = 6 Hz, CH_2 of β-Ala, 2H), 3.38 (s, CH_3O, 3H), 3.65 (s, PEG), 4.15 (q, J = 7 Hz, $CH_3C\underline{H}_2O$, 2H), 4.21 (t, J = 5 Hz, $CH_2C\underline{H}_2OC{=}ON$, 2H), 5.3 (broad, NH, 1H). Alternatively ethyl esters of urethane-linked Gly or β-Ala were prepared by reacting azeotropically dried dihydroxy-PEG or mPEG-OH with ethyl isocyanatoacetate or ethyl isocyanatopropionate (1.5 equiv. / OH) in presence of TEA (1 equiv.). Overnight reactions at 25 °C routinely resulted in quantitative conversion of the terminal hydroxyl groups (*23*).

Preparation of mPEG-O(C=O)-β-Ala-OH and its succinimidyl ester. Hydrolysis of mPEG-O(C=O)-β-Ala-OEt was performed in 2N NaOH at 25 °C for 2 h (*23,24*), or alternatively by adjusting the pH to 11 - 12 and maintaining it with dropwise addition of aqueous NaOH until the pH stabilized (*10*). The solution was acidified and the mPEG-O(C=O)-β-Ala-OH recovered by standard procedures. The succinimidyl ester was prepared using several known procedures (*25,28,50*).

Preparation of mPEG-O(C=O)-β-Ala-Hz (*7*). mPEG-β-Ala-OEt (62 g, 12 mmol) was dissolved in pyridine (120 ml) and treated with hydrazine (12 ml, 0.375 mole) under reflux for 6 h. The solution was rotary evaporated to dryness and the residue crystallized twice from *iso*-propanol and dried *in vacuo* over P_2O_5. The yield was 60 g (97 %). Colorimetric assay of hydrazide groups using TNBS gave 0.2 mmol/g (103 % of theoretical). The β-Ala content of the polymer was 0.205 mmol/g (105 % of theoretical) as determined by AAA of a completely hydrolysed (6N HCl, 110 °C, 24 h) aliquot of the product. ^{1}H-NMR ($CDCl_3$): δ 2.43 (br t, CH_2 of β-Ala, 2H), 3.38 (s, CH_3O, 3H), 3.64 (s, PEG), 4.21 (br t, $CH_2C\underline{H}_2OC{=}ON$, 2H), 5.3 (broad, NH, 1H). ^{13}C-NMR ($CDCl_3$): δ 171.2 (C=O, of Hz); 156.4 (C=O, urethane); 71.8 ($CH_3O\underline{C}H_2$); 70.0 (PEG); 68.5 ($\underline{C}H_2CH_2OC{=}O$); 63.7 ($CH_2\underline{C}H_2OC{=}O$); 58.9 ($CH_3O$); 37.1 ($NHCH_2\underline{C}H_2$); 33.9 ($NH\underline{C}H_2CH_2$) ppm. IR (neat): 3328 (NH); 1719 (C=O, urethane); 1671 (C=O, Hz) cm^{-1}.

Preparation of mPEG-carbazate. Active carbonates (nitrophenyl carbonate or

succinimidyl carbonate) or mPEG-chloroformate reacted cleanly with hydrazine to yield mPEG-O(C=O)N_2H_3, which was then purified from excess low molecular weight reagents by repeated recrystallization from ethanol.

Attachment mPEG-β-Ala-Hz to carbodiimide-activated carboxyl groups of GCSF (*7*). The mPEG-β-Ala-Hz (15.0 g, 2.9 mmol) was added to a solution of GCSF (86 mg, 4.78 mmole) in 1 mM HCl (86 ml) , followed by EDC (128 mg, 0. 667 mmol). The reaction mixture was stirred at 25 °C for 90 minutes while maintaining the pH at 4.7 - 5.0 range. Excess reagents were removed by extensive diafiltration of the reaction solution at 4 °C against 1 mM HCl. Comparison of the PEG-conjugate and native GCSF using SEC-HPLC (Zorbax GF-450 column, mobile phase was 0.2 M phosphate buffer pH 7.5) showed a substantially increased molecular weight of the conjugate. The average number of mPEG residues in the PEG-GCSF was 5.8, as determined by β-Ala quantitation by AAA. TNBS assay (*33*) confirmed that both native and PEG-modified GCSF had the same number of amino groups, indicating that the EDC activated carboxylic acid groups of the protein did not react with amino groups of the protein. The conjugate of mPEG-GCSF gave four separate bands on SDS-PAGE (PhastGel, Homogenous 12.5, Pharmacia) in the range from 29 to 67 kDa. Isoelectric Focusing (PhastGel-IEF 3-9, Pharmacia) of the conjugate resulted in the separation of six bands with pI values arranging between 6.8 and 9.0, noticeably higher than the native protein (pI 5.2; 5.9).

Preparation of HRP-PEG-Hz. HRP (40 mg, 1 μmol) and PEG-dihydrazide (320 mg, 160 μmol Hz) were dissolved in water (1 ml). EDC (50 mg) was added. Aqueous HCl (1 N) was used to maintain pH ≈ 5 for 60 min. The solution was adjusted to pH 7.5 and incubated overnight at 25 °C. The reaction solution was concentrated on Centricon-10 (Amicon), diluted with water and concentrated again. The dilution / concentration cycle was repeated until the filtrates became negative to PMA test. This indicated that all the unreacted PEG-dihydrazide was removed. When an aliquot of the purified conjugate solution was treated with PMA solution at pH 3, all the brown color of the enzyme was completely precipitated with the PEG-PMA interpolymer complex forming a clear colorless solution, suggesting that all the HRP was bound to PEG. Analysis of the HRP-PEG-Hz on SEC-HPLC (Zorbax GF-250) indicated one peak of product comparable in its elution time to a dimer of HRP (9.2 min). For comparison (HRP, MW 40kDa -10.3 min, BSA, 68kDa - 9.6 min, PEG-4kDa - 10 min). The conjugate contained on average of 3.5 Hz groups per HRP molecule as determined by TNBS assay. The conjugate exhibited binding to a Con A-Sepharose column and eluted under the same conditions as the starting HRP (15 - 20 mM methyl-α-D-mannoside). This finding was utilized in the later preparations for purification of HRP-PEG-Hz.

Conjugation of HRP-PEG-Hz to IgG. IgG (α-HCG, 3 mg) in phosphate saline buffer (0.5 ml, 50 mM Na_2HPO_4, 0.15 M NaCl, pH 5.5 adjusted with acetic acid) was treated with $NaIO_4$ (1.5 - 1.7 mg) predissolved in the same buffer (50 µl) in the dark at 4 °C for 2.5 h. The concentration of periodate in the reaction solution was 13 -15 mM. The oxidized IgG was purified by passing the reaction solution through a PD-10 column preequilibrated with the same buffer, and quickly mixed with HRP-PEG-Hz (3 mg). The conjugation solution was concentrated to ≈ 1 ml and incubated overnight at 4 °C. The reaction solution was injected into preparative SEC-HPLC (Zorbax GF-250 XL) and fractions containing the conjugate collected. At this stage the unreacted HRP-PEG-Hz was completely separated. The collected fractions were pooled and applied onto a Con A-Sepharose column equilibrated with the loading buffer (20 mM Tris · HCl, 0.3 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH 7.1). if any unconjugated IgG was present, it did not bind to the affinity column. The pure IgG-PEG-HRP was eluted from the Con A column with a gradient of methyl-α-D-mannoside (0 to 25 mM in 50 min at 1 ml/min). The ratio of HRP / IgG in the product was determined by UV / Vis calculating A_{403} / A_{280}. Typically 0.3 - 0.9 A_{403} / A_{280} values were observed indicating 1-3 HRP residues per IgG.

mPEG-hydrazone of a transaminated glycoprotein hormone. General methods for transamination of polypeptides have been described in detail elsewhere (*38,39*). For transamination of the glycoprotein hormone, the concentration of the protein was first adjusted to 1 mg/ml with 2 M sodium acetate in 0.4 M acetic acid, 0.1 M glyoxalic acid and 10 mM cupric sulfate (pH 5.5). Alternatively, the protein can be dialyzed or diafiltred into the above buffer. The reaction was allowed to proceed for two hours at room temperature. The extent of transamination was monitored by the method described by Fields and Dixon (*51*). The transaminated protein sample solution in 1 M HCl was placed in a cuvette and reacted with 2,4 - dinitrophenylhydrazine in 1 M HCl. The reaction was continuously followed by measuring the absorbtion at 370 nm until no further change was observed. The initial reading was subtracted from the final reading. The difference in absorbance was proportional to the amount of carbonyl groups on the protein molecule. The transaminated glycoprotein was subjected to AAA on an ABI 420H system (Applied Biosystems, Foster City, CA) using pre-column phenyl isothiocyanate derivatization. The transaminated protein was separated from the low molecular weight reagents by gel filtration chromatography using 100 mM sodium acetate at pH 4.5. In the second step of the reaction the transaminated protein in the elution buffer was reacted with mPEG-5k-Hz (10 to 20 mg). The PEG coupling reaction was carried out for 40 hours at room temperature. The conjugated product was purified by Sephacryl S-200 gel filtration. Three types of reactive mPEG-5k-Hz derivatives were evaluated (Table II). Of the three, the reaction between the transaminated glycoprotein and mPEG-5k-carbazate produced the best results. It was more than 95% complete. In the case of the transaminated protein coupling reaction

with mPEG-5k-hydrazide or mPEG-5k-semicarbazide the coupling reactions were only about 25% complete. The yields were improved when 0.1 % SDS was used in the reaction mixture. Gel filtration chromatography (TSK G3000SWXL column) of mPEG-carbazate-glycoprotein showed clear increase in molecular size (17.2 min) over the parent glycoprotein (18.3 min).

Preparation of immunoliposomes and peptidoliposomes. Liposomes were prepared and radiolabeled as described elsewhere (*11-13,36*). Briefly, lipid films were formed by evaporation of lipid mixtures from chloroform and hydrated with the desired aqueous phase, followed by vigorous shaking. For the ^{67}Ga-labeling the hydrating buffer contained 10 mM desferoxamine mesylate in 0.9% saline (isotonic). The resulting liposomes were extruded through polycarbonate defined pore filters to give a mean particle size of 95±15 nm, as determined by dynamic light scattering (Coulter N4SD). The typical lipid formulation contained: Hz-PEG-2k-DSPE (1.6 -5 mole %), mPEG-2k-DSPE (0-5 mole %), phosphatidyl choline (50 -60 mole %), and cholesterol (30-40 mole%). Antibodies (1-5 mg/ml), if necessary spiked with the appropriate amount of ^{125}I-IgG, were first incubated in acetate buffer (0.1M, pH 5.6) with periodate (2-20 mM) for 20-120 min. The excess of periodate was quenched by addition of NAM solution (0.1 ml at 0.5 M). Quick consumption of NAM commensurate with the amount of periodate present, presumably by conversion into Met-sulfoxide and -sulfone derivatives, was confirmed by HPLC. Finally, Hz-PEG-liposomes (1.2 ml, 20-50 µmole phospholipid / ml) were added. The mixture was left at room temperature for 1 h and then refrigerated (6 °C) until the following day. Samples were assayed for conjugation efficiency by chromatography on Sepharose 4B to separate liposome bound antibody from free IgG in 25 mM HEPES containing 15 mM NaCl, pH 7.2. The observed increase in the particle size of the immunoliposomes (diameter ≈ 120 nm), relative to the parent liposomes (≈ 100 nm) was consistent with the approximate size of IgG molecules (100Å) (*11*). Peptides containing N-terminal Ser or Thr residues were oxidized with periodate at pH 7.2 for 5 min producing N-α-glyoxylyl peptides, which were then coupled to Hz-PEG-liposomes in a similar manner (*14*). The composition of immuno- and peptido-liposomes was determined by AAA and phosphate analysis.

Abbreviations

ADH, adipic acid dihydrazide; AAA, amino acid analysis; ATP, adenosine triphosphate; AP, alkaline phosphatase; Boc, *tert*-butyloxycarbonyl; BSA, bovine serum albumine; DCC, N,N'-dicyclohexylcarbodiimide; DSPE, distearoylphosphatidylethanolamine; EDC, N-ethyl-N'-(3-dimethylamino-propyl)carbodiimide; GO, glucose oxidase; HCG, human chorionic gonadotropin; HRP, horseradish peroxidase; Hz, hydrazide; MALDI-

MS, matrix-assisted laser desorption ionization mass spectrometry ; NAM, N-acetyl methionine; PAGE, poly(acrylamide) gel electrophoresis; PEG-Nk, poly(ethylene glycol)-*molecular weight in kiloDaltons; mPEG*, monomethoxy-PEG; PMA, poly(methacrylic acid); RES, reticuloendothelial system; SC, succinimidyl carbonate; SDS, sodium n-dodecyl sulfate; SEC, size exclusion chromatography; Su, succinimide; TEA, triethylamine; TFA, trifluoroacetate; TNBS, 2,4,6-trinitrobenzene sulfonate.

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Articles

A Unique Zinc-Binding Site Revealed by a High-Resolution X-ray Structure of Homotrimeric Apo2L/TRAIL

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ABSTRACT: Apoptosis-inducing ligand 2 (Apo2L, also called TRAIL), a member of the tumor necrosis factor (TNF) family, induces apoptosis in a variety of human tumor cell lines but not in normal cells [Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C.-P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A., and Goodwin, R. G. (1995) *Immunity 3*, 673−682; Pitti, R. M., Marsters, S. A., Ruppert, S., Donahue, C. J., Moore, A., and Ashkenazi, A. (1996) *J. Biol. Chem. 271*, 12687−12690]. Here we describe the structure of Apo2L at 1.3 Å resolution and use alanine-scanning mutagenesis to map the receptor contact regions. The structure reveals a homotrimeric protein that resembles TNF with receptor-binding epitopes at the interface between monomers. A zinc ion is buried at the trimer interface, coordinated by the single cysteine residue of each monomer. The zinc ion is required for maintaining the native structure and stability and, hence, the biological activity of Apo2L. This is the first example of metal-dependent oligomerization and function of a cytokine.

Apoptosis is essential for proper metazoan development and homeostasis (*1*). Apoptosis can be initiated by a variety of trimeric TNF[1] superfamily members including TNF (or TNF-α), LT (or LTα or TNF-β), and FasL, each of which appears to have a different specific function (*2*). These trimeric molecules initiate apoptosis by binding to related receptors, some of which possess intracellular death domains. The cell killing properties of these cytokines have made them exciting targets for drug development. Unfortunately, the inflammation they trigger is too indiscriminate to make them effective therapeutics. Apo2L (or TRAIL) is a recently characterized member of the superfamily (*3, 4*). Like most TNF family members, Apo2L is a type-II transmembrane protein that can be cleaved at the cell surface to form a soluble protein (*5*). Apo2L appears to be unique among TNF-related cytokines in that it selectively induces apoptosis in tumor cells while leaving normal tissue unscathed. Administration of soluble Apo2L to mice bearing human tumors reduces tumor size with no discernible toxicity to normal tissues (*6, 7*). The resistance of normal cells to the apoptosis-inducing effects of Apo2L may be explained in part by its regulation by a family of signaling and decoy receptors (*8*). Two signaling or "death" receptors (DR4, DR5) (*9*−*11*) and three nonsignaling or "decoy" receptors (DcR1, DcR2, OPG) (*9*−*12*) for Apo2L have been identified. These decoy receptors either lack functional intracellular death domains or are shed from the cell surface. Thus, Apo2L has potential as an anti-cancer agent in humans.

Prior structural information about the TNF superfamily is derived from the X-ray structures of TNF (*13, 14*), LT (*15*), and CD40L (*16*), which reveal that these cytokines are homotrimeric jelly roll proteins. TNF family members bind the extracellular portions of cysteine-rich receptors. Ligand-induced clustering of these receptors triggers the intracellular apoptotic cascade. From the structure of LT bound to TNFR1 (*17*), it was determined that the receptors bind to ligand monomer−monomer interfaces. Recently, a low resolution structure of Apo2L confirmed its general similarity to other TNF superfamily members (*18*). In addition, we and others have determined the structure of Apo2L bound to the ecto-domain of DR5 which shows an interface centered around Apo2L residues Gln 205 and Tyr 216 (*19, 20*).

Here we present the 1.3 Å crystal structure of the Apo2L/TRAIL trimer as well as the results of an extensive mutagenesis study elucidating the receptor-binding site. The structure reveals a novel zinc-binding site, which we show is required for proper Apo2L structure and function through a comprehensive series of biochemical and biophysical experiments. The mutagenesis analysis identifies at least five

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[1] Abbreviations: Apo2L, apoptosis-inducing ligand 2; DR, death receptor; DcR, decoy receptor; LT, lymphotoxin; OPG, osteoprotegerin; rmsd, root-mean-square deviation; TNF, tumor necrosis factor; TNFR1, TNF p55 receptor; ICP-AES, inductively coupled plasma atomic emission spectrometry.

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residues, distributed in two distinct patches, that are critical for high-affinity receptor binding and activity.

MATERIALS AND METHODS

Expression and Purification of Apo2L Mutants. Fragments of the extracellular domain of Apo2L corresponding to residues 91−281 or 114−281 were expressed in *Escherichia coli* and purified as previously described (*7*). Both fragments contain the entire region that is homologous to the receptor binding domain of TNF (Figure 2). The two fragments are equivalent for receptor binding, but the shorter form (114−281) is 1.5-fold more bioactive. Alanine substitution mutants of Apo2L were constructed by oligonucleotide-directed mutagenesis (*21*) of a plasmid (pAPOK5) designed for the intracellular *E. coli* expression of Apo2L (91−281) under control of the *trp* promoter. *E. coli* strain 294 transformed with the mutated plasmids were grown to mid-log phase at 37 °C in 250 mL of M9 media supplemented with 100 μM $ZnSO_4$ (no $ZnSO_4$ was added to the media for the production of protein used for crystallography), expression was induced by the addition of 25 μg/mL β-indole acrylic acid, and the cultures were grown overnight at 30 °C. Cells were harvested by centrifugation and frozen. The cell pellet was homogenized in 6 vol of 0.1 M Tris-HCl, pH 8, 0.2 M NaCl, 5 mM EDTA, and 5 mM DTT, and Apo2L was isolated from the soluble fraction by IMAC on a chelating hiTRAP column (Pharmacia) charged with nickel. Although the protein was not expressed with a histidine tag, Apo2L by itself has a weak affinity for immobilized metal and can be eluted with low concentrations of imidazole. A final purification was obtained by cation-exchange chromatography on an SP hiTRAP column (Pharmacia). Concentrations of purified Apo2L mutants were determined by absorbance measurements using an ϵ_{280} of 1.4 mg^{-1} mL cm^{-1}.

Crystallography. Crystals of wild-type Apo2L (114−281) were grown at 20 °C in 70 μL of sitting drops consisting of 40 μL of protein (at 2.6 mg/mL in 20 mM Tris, pH 8.0); 20 μL of 50 mM Tris, pH 8.0; and 10 μL of 8% poly(ethylene glycol) (PEG) 2K MME over a well solution of 50% PEG 2K MME. The space group was determined to be $P6_3$ with two monomers in the asymmetric unit and cell constants of $a = 72.5$ Å and $c = 140$ Å. These crystals are similar, but not identical, to Apo2L crystals reported recently (*22*). Crystals of Apo2L mutant Asp 218 Ala (residues 91−281 with Asp 218 mutated to alanine) grew at 4 °C in 14 μL of sitting drops containing 4 μL of 4% MPD and 10 μL of protein (1.7 mg/mL in 20 mM Tris-HCl, pH 7.5) over a well solution of 32% MPD. The space group was *R*32 with one monomer per asymmetric unit and cell parameters of $a = 66.4$ Å and $c = 197.7$ Å, and the crystals diffracted to a resolution of 1.3 Å at −180 °C with synchrotron radiation. Data sets to 3.9 Å resolution for the wild-type crystals and to 1.9 Å for the mutant were measured on a Rigaku rotating anode X-ray generator equipped with a MAR detector and processed with the program HKL (*23*). A 1.3 Å data set of the mutant was measured at the Structural Biology Center at Argonne National Labs and processed with HKL, giving $R_{merge} = 6.4\%$ (34% in the 1.35−1.30 Å shell), 100% completeness, 12-fold redundancy, and $\langle I/\sigma I \rangle = 12.4$. The wild-type structure was solved by molecular replacement using the program Amore (*24*) and a model based on TNF (PDB code 1TNF) and was refined (*25*) to an R_{free} of 33.5%. The mutant was initially refined against the 1.9 Å data set to an R_{free} of 25% and subsequently against the 1.3 Å data using Refmac (*23*) and SHELXL (*26*). During refinement, a 28σ peak of difference electron density was observed on the 3-fold axis near Cys 230 and modeled as a zinc ion; its B-factor refined to 11.8 $Å^2$. A fourth zinc ligand was identified on the trimer axis and refined as chloride ($B = 14.3$ $Å^2$). This ligand was tentatively identified as chloride after placing a water molecule at this position resulted in a refined B-factor of 4.0 $Å^2$ with a residual 7σ peak of difference electron density. The R and R_{free} are 14.1% and 19.7%, respectively, and the model has good stereochemistry with rms differences in bond lengths and angles of 0.014 Å and 2.54°. The final model consists of residues 120−130, 142−194, and 197−281 with 226 water molecules, one zinc ion, and one chloride ion. N-terminal sequencing and mass spectrometry analysis show that the protein used for crystallization was intact; therefore, the missing residues are presumably disordered. The coordinates for the Apo2L Asp 218 Ala structure have been deposited with the Protein Data Bank (access code 1DG6). Figures 1 and 4 were made with the programs Molscript (*27*) and Raster3D (*28*).

Determination of Receptor Binding Affinity. Dissociation constants (K_D) for the binding of Apo2L mutants to immobilized receptor immunoadhesions were determined from surface plasmon resonance measurements on a Pharmacia BIAcore 2000 (Pharmacia Biosensor, Piscataway, NJ). The receptor proteins were coupled to the sensor chip surface at a level of 500−1000 resonance units using the amine coupling chemistry (Pharmacia Biosensor). Sensorgrams were recorded for Apo2L binding at concentrations ranging from 15.6 to 500 nM in 2-fold increments. The kinetics constants were determined by nonlinear regression analysis according to a 1:1 binding model using software supplied by the manufacturer. Binding constants were calculated from the kinetic constants.

Bioactivity of Apo2L Mutants in Vitro. A bioassay that measures cell viability from the metabolic conversion of a fluorescent dye was used to determine the apoptotic activity of Apo2L variants. Serial 2-fold dilutions of wild-type or mutant Apo2L were made in RPMI-1640 media (Gibco) containing 0.1% BSA, and 50 μL of each dilution was transferred to individual wells of 96-well Falcon tissue culture microplates. (The formula for RPMI-1640 media does not include a zinc salt. The zinc concentration in this media is less than the detection limit for ICP-AES.) A total of 50 μL of SK-MES-1 human lung carcinoma cells (ATCC-CHTB58) (in RMPI-1640, 0.1% BSA) was added at a density of 2×10^4 cells/well. These mixtures were incubated at 37 °C for 24 h. At 20 h, 25 μL of alamarBlue (AccuMed, Inc., Westlake, OH) was added. Cell number was determined by measuring the relative fluorescence at 590 nm upon excitation at 530 nm. These data were analyzed by using a four-parameter fit to calculate ED_{50}, the concentration of Apo2L giving a 50% reduction in cell viability.

Metal Content of Apo2L. Elemental analysis of Apo2L was performed using inductively coupled plasma atomic emission spectrometry (ICP-AES) (*29*). The levels of Cd, Co, Zn, Ni, and Cu were determined in a 2 mg/mL solution of Apo2L (114−281) formulated in 20 mM Tris, pH 7.5, as well as in the formulation buffer. The standard error for these determinations was ±5%. For removal of the bound metal, a

sample of Apo2L was sequentially dialyzed versus 50 mM EDTA/2 mM 1,10-phenanthroline, and then versus metal-free 20 mM Tris, pH 7.5. After this treatment, a majority of the Apo2L migrated as a disulfide-linked dimer upon SDS−PAGE. For further analysis, the zinc-depleted Apo2L was reduced by incubation with 10 mM DTT for 1 h at ambient temperature followed by desalting on a PD-10 column (Pharmacia) equilibrated with PBS. Analysis by SDS−PAGE suggested complete reduction; however, the disulfide bonds were observed to slowly reform over a period of a few days at ambient temperature.

Circular Dichroic Spectroscopy. CD spectra were recorded on an Aviv model 202 circular dichroic spectrometer (Aviv, Inc.). Spectra were acquired at 25 °C on 20 μM solutions of protein, prepared in PBS, by using a step size of 0.5 nm and averaging time of 20 s. Quartz, rectangular cuvettes having a path length of 0.1 cm were used. Thermal denaturation experiments were performed using the same cuvettes and protein solutions. Measurements were made at 2 °C intervals following a 1-min equilibration period at the specified temperature. CD was measured at 222 nm using a 20-s averaging time. Simultaneously, fluorescence was recorded using a photomultiplier placed at a right angle to the CD path. The 222-nm light was used as the excitation source, and a 320-nm cutoff filter was placed in front of the photomultiplier tube to minimize stray light detection. The protein solutions were turbid upon cooling to ambient temperature, suggesting irreversible denaturation, and thus a rigorous thermodynamic analysis of the data was not performed.

Fluorescence Spectroscopy. Tryptophan fluorescence emission spectra were recorded on a SLM8000 spectrofluorimeter (SLM Instruments, Inc.). Spectra were obtained by using 1 μM protein solutions in PBS and rectangular quartz cells having a path length of 1 cm. Excitation was at 295 nm, and the emission spectrum was scanned from 305 to 400 nm using a 0.5-nm step size and a 1.0-s integration time.

RESULTS AND DISCUSSION

The X-ray structure of Apo2L was determined by molecular replacement using a model of TNF (*14*) and refined to 3.9 (wild type) and 1.3 Å (Asp 218 Ala mutant). Like other members of the TNF family, and as also confirmed by the low-resolution structure (*18*), Apo2L is a compact trimer formed of three jelly roll monomers that bury approximately 5100 Å^2 (1700 Å^2 per monomer) of solvent-accessible surface to form the globular trimer (Figure 1A). The structure is well defined, with the exception of a disordered N-terminal extension and two partially disordered loops. As expected from a low-resolution structure of Apo2L (*18*), the position of the core β-strands is well conserved as compared to the other structurally characterized members of the TNF family, TNF (*13, 14*), LT (*15*), and CD40L (*16*), with a rms difference of 0.8 Å when superimposed on the strands of TNF or LT. Even though all TNF family members are expected to form trimers, none of the residues in the trimer interface are absolutely conserved; nevertheless, the hydrophobic character of the interface residues is preserved. The known structures are most similar at the top (the widest part of the trimer) and along the upper half of the 3-fold axis. Near the tip of the trimer interface, in the vicinity of Cys 230, the structures diverge considerably, especially in the conformation of the 190s and 230s loops.

In contrast to the invariant β-scaffold core, the structure of the loops and receptor binding surfaces varies significantly among the TNF family members. One major difference between the structures of Apo2L on one hand and TNF, LT, and CD40L on the other is the connection between strands A and A′. In TNF, LT, and CD40L, strand A is followed by a compact loop (see Figure 2). In Apo2L, a 15-residue insertion drastically lengthens this loop and alters its conformation. The first part of the loop (residues 131−141) is disordered while the second part (residues 142−154) crosses the surface of the molecule from one monomer−monomer interface to the next (Figure 1A) and has a conformation that resembles CD40L in its C-terminal portion.

A Novel Zinc-Binding Site Is Required for Structure and Function. An unanticipated finding in this structure is the presence of a novel zinc-binding site, buried near the tip of the trimerization interface. The three symmetry-related Cys 230 residues point inward toward the trimer axis and coordinate a metal ion in conjunction with an interior solvent molecule (possibly a chloride ion). This metal binding site exhibits a slightly distorted tetrahedral geometry with bonds and angles appropriate for a zinc-binding site and is completely inaccessible to solvent (Figure 1B). The identity of the bound metal was confirmed using inductively coupled plasma atomic emission spectrometry (ICP-AES (*29*)). In a quantitative analysis for Cd, Co, Zn, Ni, and Cu, 0.79 mol of Zn and 0.06 mol of Co per mol of Apo2L trimer were detected, showing that the bound ion in this structure is zinc with a stoichiometry of approximately one metal ion per trimer. Since zinc was not present in either the purification or crystallization buffer, the source must have been the *E. coli* intracellular pool or the growth media.

The functional importance of the zinc-binding site is demonstrated by the observation that alanine or serine substitution of Cys 230 results in 20- and 70-fold reductions in apoptotic activity, respectively (Table 1). Furthermore, removal of the bound metal from wild-type Apo2L by dialysis against chelating agents results in a significant decrease in receptor-binding affinity (Table 1) and a 90-fold decrease in apoptotic activity (Figure 3). Interestingly, upon removal of the zinc, the cysteines become prone to oxidation, and Apo2L forms poorly active, disulfide-linked dimers. Although native Apo2L can also form disulfide-linked dimers upon extended storage in the absence of reducing agents, the rate of oxidation is much faster for zinc-depleted Apo2L. Therefore, metal binding appears to play an indirect role in receptor binding and apoptotic activity since the zinc-binding site is buried and is not expected to contact the receptor. The variability in effect on apoptosis resulting from zinc depletion or Cys230 replacement (20−90-fold) probably reflects the energetic cost of burying a nonpolar residue (Ala) versus a polar residue (Ser or unliganded Cys).

Spectroscopic and melting temperature characterization of Apo2L show that zinc binding is important for maintaining the structure and stability of the Apo2L trimer. Far-ultraviolet CD spectra (Figure 4A) suggest a diminished β-sheet content for zinc-depleted Apo2L. Similarly, zinc-depleted Apo2L displays a 2.2-fold decreased tryptophan fluorescence relative to native Apo2L (Figure 4B). The decrease in fluorescence is consistent with a conformational change of Apo2L upon

FIGURE 1: Overall structure of Apo2L. (A) View of the trimer perpendicular to the 3-fold axis. The ordered protein commences at residue 120. The two disordered, or marginally ordered, loops, residues 131−141 and 194−202, are marked with dashed lines. The zinc-binding site including the three symmetry-related cysteines and a putative chloride ligand are shown in space filling rendering. The sulfur atoms are yellow, the zinc is orange, and the chloride is pink. (B) Wall-eyed stereoview of the zinc-binding site; the angles between Sγ-zinc-Sγ are 114° and the Sγ-zinc-chloride angles are 107°, with 2.3 Å zinc-to-ligand bond distances.

loss of zinc resulting in increased solvent exposure of Trp 231, which is adjacent to the zinc-binding site (Figure 1B). The only other tryptophan residue in Apo2L, Trp 154, which is conserved among TNF family members (Figure 2), is buried in the core of the monomer far from the zinc-binding site and should be insensitive to the occupancy of the zinc-binding site. Addition of $ZnSO_4$ to the solution of zinc-depleted Apo2L results in a partial restoration of the tryptophan fluorescence (Figure 4B), suggesting that the conformational changes resulting from zinc removal are reversible. Conditions of zinc concentration, time, and temperature leading to a full recovery of fluorescence have not been determined. Thermal denaturation experiments show that zinc binding is important for the stability of Apo2L (Figure 4C). Both proteins display a cooperative unfolding transition, as detected by circular dichroism at 222 nm, but the melting temperature for zinc-depleted Apo2L is about 25 °C lower than measured for native Apo2L. Thermal denaturation of both proteins was irreversible, as a consequence of disulfide bond formation and aggregation, and thus ΔG values for unfolding were not determined. A cooperative thermal transition, coincident with the changes in circular

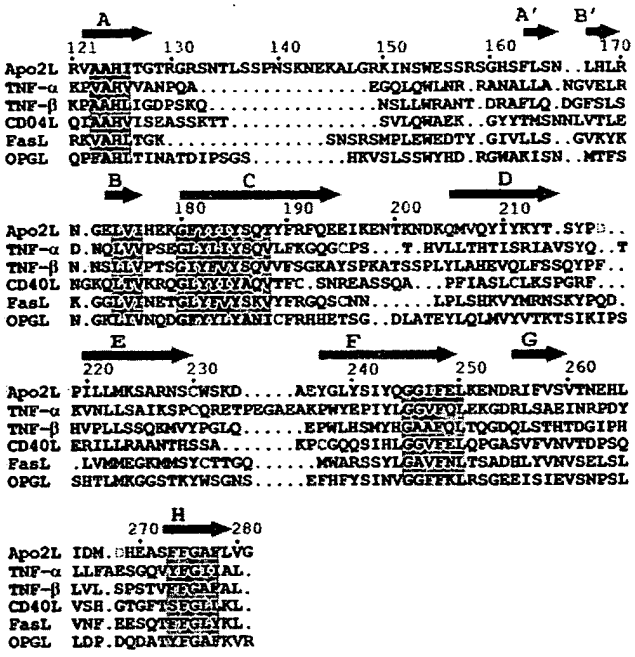

FIGURE 2: Sequence alignment of selected TNF family members. Residues that when mutated to alanine have a greater than 5-fold decrease on bioactivity are shown in red, residues that resulted in increased bioactivity are in purple, and cysteines are in green. Arrows over the sequence indicate β-strands in Apo2L. Conserved sections of the sequence are highlighted in blue. Numbering over the sequences refers to Apo2L sequence.

dichroism, is also observed by fluorescence measurements for native but not zinc-depleted Apo2L. These data indicate that removal of the zinc may cause an initial local unfolding of Apo2L that immediately exposes Trp 231 to solvent and also destabilizes the protein resulting in the shift in the melting temperature.

Since the zinc-binding site is located on the trimer axis, it is anticipated that the presence of zinc influences the trimerization constant. At the concentration limits of detection for dynamic light scattering and gel filtration monitored by absorbance at 280 nm, about 10 μM, both native and zinc-depleted Apo2L gave equivalent molecular weights consistent with a trimer (data not shown). Given the extensive monomer−monomer contact observed in Apo2L that is similar to other TNF family members, it is expected that zinc-depleted Apo2L is a relatively tight trimer. Indeed, gel filtration experiments with radiolabeled TNF, which does not have a zinc-binding site, show that the TNF trimer does not dissociate at concentrations as low as 10 nM (*30*). TNF also retains cytotoxic activity at concentrations less than 1 nM. Nonetheless, an increase in the trimer dissociation constant probably contributes to the lower thermal stability and decreased bioactivity measured for zinc-depleted Apo2L.

The Apo2L structure represents the first example of metal binding-mediated trimerization of a cytokine. This zinc-binding site is essential for full bioactivity. While zinc-binding sites have been shown to play structural roles in protein−protein interactions in many proteins involving diverse interfaces (*31*−*35*), none of the other structurally characterized members of the TNF family (CD40L, TNF, or LT) have metal-binding sites. On the basis of sequence analysis, the TNF family can be divided into three groups with respect to Cys 230 (see Figure 2). Some family members, such as LT and osteoprotegerin ligand, do not have a cysteine residue corresponding to Cys 230 of Apo2L. A second group, represented by TNF and Fas ligand, is characterized by the presence of a second cysteine residue in the loop adjacent to Cys 230 (the 194−203 loop in Apo2L), resulting in the formation of a disulfide bridge and precluding the formation of a metal-binding site. Finally, the only family members with an unpaired Cys 230 residue are Apo2L and its only known ortholog, murine Apo2L. In this regard, it is noteworthy that the conformation of the main chain immediately prior to Cys 230 in Apo2L differs from the disulfide-containing family members such as TNF and CD40L: in Apo2L, the side chain of Cys 230 is oriented toward the interface instead of away from it. Thus, the zinc-binding site confers unique structural and stability features to Apo2L differentiating it from all other TNF family members.

Table 1: Receptor Binding and Apoptotic Activity of Apo2L Mutants[a]

	ratio (mutant/wild-type)			
mutant	DR4-IgG K_D	DR5-IgG K_D	DcR2-IgG K_D	apoptosis ED_{50}
Δzinc	6.3	6.6	11.2	90.0
R130A	3	2.7	1.3	1.9
N134A	1.0	0.8	1.0	1.5
L136A	3.3	1.5	1.4	0.8
S138A	2.1	1.3	2.2	1.2
N140A	1.4	1.9	0.9	1.1
S141A	2.3	1.3	2.4	1.3
K142A	2.6	1.9	2.7	2.0
N143A	2.1	2.0	1.3	1.5
R149A	1.8	2.2	1.6	3.5
S153A	2.3	1.2	2.1	0.9
E155A	1.6	2	1.4	2.5
R158A	2.4	1.3	6.5	1.4
S159A	4.7	2.2	3.4	0.9
R170A	1.1	2.2	0.6	0.9
K179A	0.9	0.9	1.1	2.0
R191A	7.8	3.9	3.2	2.2
Q193A	1.7	1.1	1.2	2.2
E195A	4.6	1.4	2.6	0.8
K197D	2	2.1	2.9	1.1
K201A	4.3	2.7	10	2.5
N202A	2.5	2.5	1.9	3.2
D203A	1.5	1.1	0.6	0.5
Q205A	13.1	6.3	10.8	690
V207A	2.2	2.8	2.1	5.6
Y213A	1.3	1	1.5	1.2
Y216A	14.5	8.9	9.0	320
D218A	1.3	1.9	1.1	0.3
C230A	4.1	7.1	6.7	20
C230S	5.3	3.4	9.0	70
E236A	6.0	9.8	8.4	10.8
Y237A	7.3	5.0	48	8.3
Y240A	1.8	0.8	1.8	1.1
K251A	1.9	2	2.4	0.8
S259A	4.3	2.0	1.4	3.3
H264A	1.9	2.0	1.4	3.1
D267A	5.7	1.9	5.5	1.1
D269A	1.7	0.5	0.9	0.2

[a] Values shown represent the ratio of mutant to wild type. For wild-type Apo2L (91−281), the K_D values for DR4-IgG, DR5-IgG, and DcR2-IgG are 0.8 ± 0.3, 0.9 ± 0.4, and 0.3 ± 0.2 nM. The K_D measured for DR5 is consistent with results reported by Emery et al. (*12*). Wild-type Apo2L (91−281) gave an ED_{50} of 24.0 ± 3.1 ng/mL in the apoptosis assay while the 114−281 form of wild-type Apo2L was slightly more active and gave an ED_{50} of 16.0 ± 3.6 ng/mL. Only 2-fold and greater changes from wild-type values are considered to be significant.

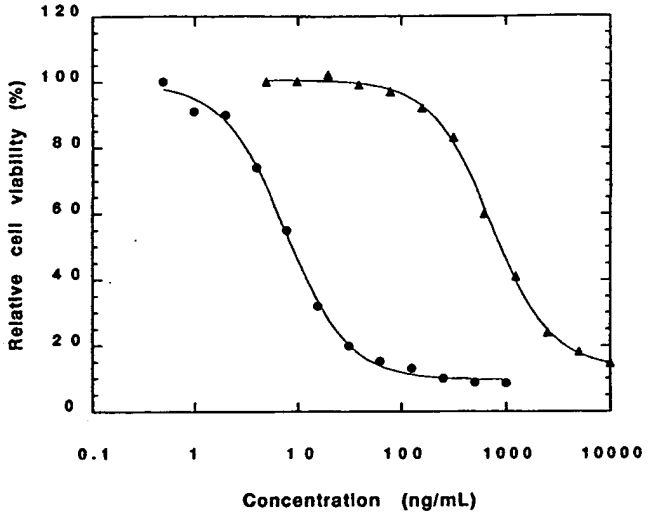

FIGURE 3: Bioassay data showing the functional importance of the zinc-binding site. Cell (SK-MES-1) viability was determined by a fluorescence assay of metabolic activity after overnight incubation with varying concentrations of wild-type Apo2L (114−281) (●) and wild-type Apo2L (114−281) treated with chelating agents to remove the zinc (▲).

Structural Effects of the Removal of Zinc. A comparison of our present structure to the three other known structures of Apo2L/TRAIL, namely, the low-resolution structure without zinc (*18*), the structure without zinc and bound to DR5 (*20*), and that of the complex with DR5 and containing zinc (*19*), reveals the effects of removing the zinc ion on the local structure of Apo2L. In the absence of zinc (*18*), the six residues following Cys 230 (including Trp 231) are disordered, and Cys 230 is described as being only 2.6 Å from its symmetry-related copies. In the zinc-depleted structure of the DR5-Apo2L complex, a disulfide bond is proposed to exist between two of the three symmetry-related cysteines (*20*). In addition, the 230s loop is very poorly ordered, as evidenced by the high-temperature factors in this segment (for example, the average B-factor for Cys 230 and Trp 231 is 100 and 142 Å^2, respectively, compared to an average of 50 Å^2 for the entire structure). The disorder in this loop in the zinc-depleted structures is consistent with our tryptophan fluorescence data, indicating that in the absence of zinc the Trp 231 side chain is likely solvent exposed (Figure 4B). In contrast, in the present, zinc-bound structure, the 230s loop is highly ordered with an average B-factor of only 36 Å^2, or about 10 Å^2 below the overall average. Furthermore, the conformation of this loop differs grossly from that observed in the absence of zinc, resulting in a buried and closely packed environment of Trp 231 in the zinc-bound trimer. It is noteworthy that the changes in loop conformation are propagated to Glu 236, resulting in changes in the receptor interactions for this side chain. In summary, the removal of zinc from Apo2L results in large conformational changes in the region near Cys 230, including potential mixed disulfide-bonded trimers and a partially exposed position of the side chain of Trp 231, which extend to the periphery of the receptor-binding site. The result of these changes may therefore include direct effects on receptor binding in addition to the marked destabilization of the structure revealed by our melting experiments.

The Receptor-Binding Site Shows Two Functionally Important Clusters of Residues. To map the receptor-binding site, amino acid residues important for binding and biological activity were identified by alanine-scanning mutagenesis (*36*). Residues were chosen for substitution on the basis of solvent accessibility and by analogy to the receptor contacts observed in the structure of the complex between LT and TNFR1 (*17*). Single alanine substitutions at five positions, residues Gln 205, Val 207, Tyr 216, Glu 236, or Tyr 237, resulted in a greater than 5-fold decreases in apoptotic activity in a bioassay (Table 1, Figure 5). Four of these five critical alanine substitutions (Gln 205, Tyr 216, Glu 236, and Tyr 237) resulted in at least a 5-fold decreased affinity for DR4, DR5, and DcR2. Of these residues, Gln 205 and Tyr 216 are the most critical in induction of apoptosis since alanine substitution at these two sites results in a greater than 300-

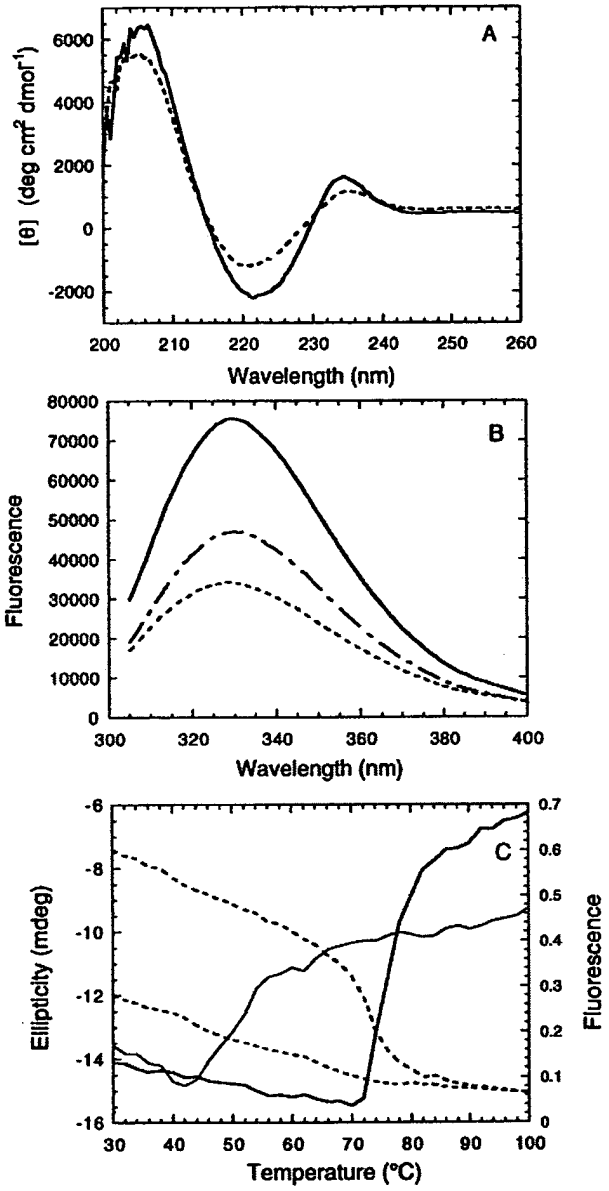

FIGURE 4: Effect of zinc removal on the conformation and stability of Apo2L. (A) Far-ultraviolet circular dichroic spectra of native (solid line) and zinc-depleted (dashed line) Apo2L. (B) Tryptophan fluorescence emission spectra of native (solid black line) and zinc-depleted (dashed red line) Apo2L. The spectrum observed after a 4-h ambient temperature incubation of zinc-depleted Apo2L with 50 μM $ZnSO_4$ is shown as the dot−dashed blue line. (C) Thermal denaturation of native (blue) and zinc-depleted (red) Apo2L. CD at 222 nm is shown as the solid lines, and fluorescence emission above 320 nm is shown as the dashed lines.

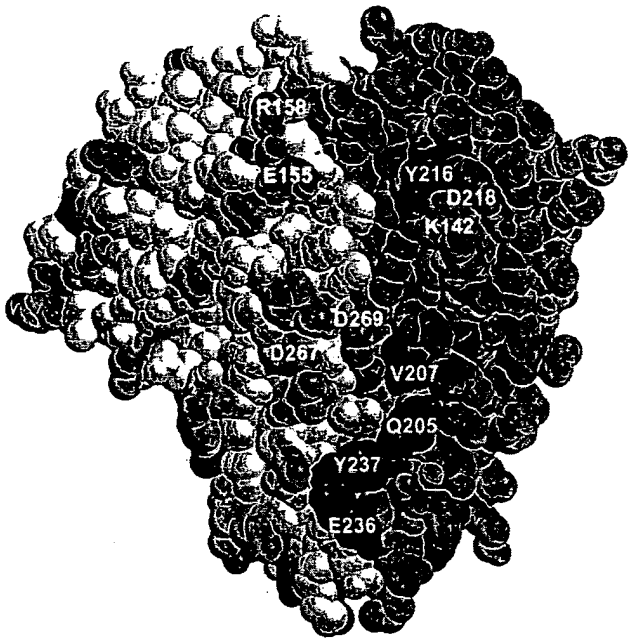

FIGURE 5: Mutational analysis of Apo2L. The trimer is oriented approximately as in Figure 1. Residues with a greater than 5-fold decrease or 2-fold increase in bioactivity when mutated to alanine are shown in red and yellow, respectively. Other residues mutated in this study are shown in cyan.

fold reduction in bioactivity. The mutant proteins with significantly reduced apoptotic activity also have impaired binding to both DR4 and DR5, suggesting that binding to at least one of these receptors is required for the biological effect.

In addition to the residues critical for apoptosis, many other alanine substitutions lead to small decreases (2−5-fold) in receptor-binding and apoptotic activity. Furthermore, alanine substitutions of Asp 218 or 269 resulted in 3−5-fold increases in apoptotic activity although neither mutation significantly affected receptor binding. Most of the minor deleterious alanine substitutions have similarly impaired binding to both DR4 and DR5, the only exceptions being mutation of Glu195 and Asp267, which have a pronounced effect on DR4 binding but a negligible effect on DR5 binding. Neither the E195A or D267A mutants of Apo2L have decreased apoptotic activity, suggesting that DR5 binding alone may be sufficient to induce apoptotic death in SK-MES-1 lung carcinoma cells upon Apo2L administration. Changes in DcR2 binding tend to parallel the effects observed for DR4 binding with the exception that alanine substitutions of Arg158 and Tyr237 have larger effects on DcR2 binding. Overall, these data suggest a similar contact region on Apo2L for DR4, DR5, and DcR2.

When the results of the mutagenesis analysis are mapped onto the structure, the functional epitope of Apo2L for receptor binding and biological activity is located along the walls of a surface crevice formed by adjoining monomers that runs from the wider part of trimer to the variable loops at the tip (Figure 5). This epitope is divided into two groups of residues: a concentrated patch of residues at the tip of the crevice and several other residues spread along the surface of the crevice at the wider part of the trimer. Each group provides an essential residue for signaling cell death, Tyr 216 at the top and Gln 205 at the tip of the trimer. Contact with these two residues appears to be crucial for organization of the receptors in the signaling complex since alanine substitution at these sites has a greater effect on apoptosis-inducing activity than on receptor binding. Apo2L residues Glu 155, Arg 158, and Tyr 216, at the top of the trimer, structurally correspond to TNF residues Leu 29, Arg 32, and Tyr 87, which have been shown to be critical for TNF receptor interactions (*37*−*39*). Similarly, residues in LT (*39*) and CD40L (*16*) important for receptor binding are also located on the wider part of the trimer. Many of these residues (Glu155 and Arg 158 in Apo2L, Asp 50 in LT, Arg 132 in TNF, and Lys 143 and Tyr 145 in CD40L) map to the C-terminal portion of the AA′ loop between strands A and A′. In contrast, the residues in TNF, LT, or CD40L that correspond to Apo2L residues Gln 205, Val 207, Glu 236, and Tyr 237 are not functionally important in these cytokines. In Apo2L these residues are crucial for receptor binding and biological activity and are located near the tip of the trimer in the more variable part of the structure (Figure 5). The result of these differences is that, while for TNF, LT, and CD40L the upper half of the trimer makes the most important contribution to receptor binding, additional key binding residues of Apo2L are found near the tip of the trimer, in the vicinity of the zinc-binding site. Therefore, the structural analysis of the mutagenesis data suggests that Apo2L has a larger and more extended contact surface for interaction with its target receptors than CD40L, TNF, or LT. The contact surface predicted by alanine scanning mutagenesis is in very good agreement with the interface observed in a crystal structure of Apo2L bound to DR5 (*19*).

In contrast to the well-characterized interaction between human growth hormone and its receptor (*40*), Apo2L does not present a "hot spot" epitope in which a few residues make a dominant contribution to receptor binding. None of the Apo2L alanine substitutions cause a greater than 20-fold reduction in affinity for DR4 or DR5, and the binding energy for Apo2L−receptor interaction appears to be more evenly distributed within the extended contact surface. This could be a consequence of Apo2L having evolved to recognize multiple receptors. Concentration of binding energy in a few residues might be incompatible with broad specificity for several receptors. One implication of these findings is that it may be very difficult to produce small molecule Apo2L mimetics since the overall binding interaction is built from multiple, weak interactions. Conversely, it may be possible to exploit the quantitative differences in receptor affinity shown here (Table 1) to engineer receptor selective variants of Apo2L. For example, a variant with greatly decreased DcR2 binding but only slightly diminished DR5 binding might be produced by combining substitutions at 158, 201, and 237. Receptor selective variants of Apo2L should be useful for further studies of the biological mechanism of action of this cytokine and might have interesting therapeutic profiles differing from those of the wild-type protein.

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